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## (54) Compositions and methods for screening drug libraries

(57) A method of screening for binding partners of a specific molecule. The method employs a chimeric protein having at least two different binding regions; one containing at least a portion of the specific molecule or

an analog thereof, and the other containing a binding region of an immunoglobulin chain. In a preferred embodiment, the method is used for rapidly screening member compounds of a combinatorial library for potential biological activity.

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**Description****Field of the Invention**

5        The present invention relates to the fields of chemistry, molecular biology and biochemistry. The invention relates to methods for identifying, from a large collection of random or non-random synthetic molecules, candidates of such molecules able to bind a specific domain of a target molecule. The invention therefore has useful applications in fields including basic biochemical and biomedical research and drug development.

**Background of the Invention**

A significant recent development in pharmaceutical drug discovery and design has been the development of combinatorial chemistry to create chemical libraries of potential new drugs. Chemical libraries are intentionally created collections of different molecules; these molecules can be made by organic synthetic methods or biochemically. In the latter case, the molecules can be made *in vitro* or *in vivo*.

15      Combinatorial chemistry is a synthetic strategy in which the chemical members of the library are made according to a systematic methodology by the assembly of chemical subunits. Each molecule in the library is thus made up of one or more of these subunits. The chemical subunits may include naturally-occurring or modified amino acids, naturally-occurring or modified nucleotides, naturally-occurring or modified saccharides or other molecules, whether organic 20 or inorganic. Typically, each subunit has at least two reactive groups, permitting the stepwise construction of larger molecules by reacting first one then another reactive group of each subunit to build successively more complex and potentially diverse molecules.

25      By creating synthetic conditions whereby a fixed number of individual building blocks, for example, the twenty naturally-occurring amino acids, are made equally available at each step of the synthesis, a very large array or library of compounds can be assembled after even a few steps of the synthesis reaction. Using amino acids as an example, at the first synthetic step the number of resulting compounds ( $N$ ) is equal to the number of available building blocks, designated as  $b$ . In the case of the naturally-occurring amino acids,  $b = 20$ . In the second step of the synthesis, assuming that each amino acid has an equal opportunity to form a dipeptide with every other amino acid, the number of possible compounds  $N = b^2 = 20^2 = 400$ .

30      For successive steps of the synthesis, again assuming random, equally efficient assembly of the building blocks to the resulting compounds of the previous step,  $N = b^x$  where  $x$  equals the number of synthetic assembly steps. Thus it can be seen that for random assembly of only a decapeptide the number of different compounds is  $20^{10}$  or  $1.02 \times 10^{13}$ . Such an extremely large number of different compounds permits the assembly and screening of a large number of diverse candidates for a desired enzymatic, immunological or biological activity.

35      Biologically synthesized combinatorial libraries have been constructed using techniques of molecular biology in bacteria or bacteriophage particles. For example, U.S. Patents No. 5,270,170 and 5,338,665 to Schatz describe the construction of a recombinant plasmid encoding a fusion protein created through the use of random oligonucleotides inserted into a cloning site of the plasmid. This cloning site is placed within the coding region of a gene encoding a DNA binding protein, such as the lac repressor, so that the specific binding function of the DNA binding protein is not 40 destroyed upon expression of the gene. The plasmid also contains a nucleotide sequence recognized as a binding site by the DNA binding protein. Thus, upon transformation of a suitable bacterial cell and expression of the fusion protein, the protein will bind the plasmid which produced it. The bacterial cells are then lysed and the fusion proteins assayed for a given biological activity. Moreover, each fusion protein remains associated with the nucleic acid which encoded it; thus through nucleic acid amplification and sequencing of the nucleic acid portion of the protein:plasmid 45 complexes which are selected for further characterization, the precise structure of the candidate compound can be determined. The Schatz patents are incorporated herein by reference.

50      In other biological systems, for example as described in Goedell *et al.*, U.S. Patent No. 5,223,408, nucleic acid vectors are used wherein a random oligonucleotide is fused to a portion of a gene encoding the transmembrane portion of an integral protein. Upon expression of the fusion protein it is embedded in the outer cell membrane with the random polypeptide portion of the protein facing outward. Thus, in this sort of combinatorial library the compound to be tested is linked to a solid support, *i.e.*, the cell itself. A collection of many different random polypeptides expressed in this way is termed a display library because the cell which produced the protein "displays" the drug on its surface. Since the cell also contains the recombinant vector encoding the random portion of the fusion protein, cells bearing random polypeptides which appear promising in a preliminary screen can be lysed and their vectors extracted for nucleic acid 55 sequencing, deduction of the amino acid sequence of the random portion of the fusion protein, and further study. The Goedell patent is incorporated herein by reference.

Similarly, bacteriophage display libraries have been constructed through cloning random oligonucleotides within a portion of a gene encoding one or more of the phage coat proteins. Upon assembly of the phage particles, the random

polypeptides also face outward for screening. As in the previously described system, the phage particles contain the nucleic acid encoding the fusion protein, so that nucleotide sequence information identifying the drug candidate is linked to the drug itself. Such phage expression libraries are described in, for example, Sawyer *et al.*, 4 *Protein Engineering* 947-53 (1991); Akamatsu *et al.*, 151 *J. Immunol.* 4651-59 (1993), and Dower *et al.*, U.S. Patent No. 5,427,908.

5 These patents and publications are incorporated herein by reference.

While synthesis of combinatorial libraries in living cells has distinct advantages, including the linkage of the compound to be tested with a nucleic acid capable of amplification by the polymerase chain reaction or another nucleic acid amplification method, there are clear disadvantages to using such systems as well. The diversity of a combinatorial library is limited by the number and nature of the building blocks used to construct it; thus modified or R-amino acids 10 or atypical nucleotides may not be able to be used by living cells (or by bacteriophage or virus particles) to synthesize novel peptides and oligonucleotides. There is also a limiting selective process at play in such systems, since compounds having lethal or deleterious activities on the host cell or on bacteriophage infectivity or assembly processes will not be present or may be negatively selected for in the library. Importantly, only peptide or oligonucleotide compounds are made in such systems; thus the diversity of the library is restricted to peptide and polynucleotide macromolecules 15 composed of naturally-occurring monomeric units.

Other approaches to creating molecularly diverse combinatorial libraries employ chemical synthetic methods to make use of atypical or non-biological building blocks in the assembly of the compounds to be tested. Thus, Zuckermann *et al.*, 37 *J. Med. Chem.* 2678-85 (1994), describe the construction of a library using a variety of N- (substituted) glycines for the synthesis of peptide-like compounds termed "peptoids". The substitutions were chosen to provide a series of aromatic substitutions, a series of hydroxylated side substitutions, and a diverse set of substitutions including branched, amino, and heterocyclic structures. This publication is incorporated by reference herein.

20 Other workers have used small bi- or multifunctional organic compounds instead of, or in addition to, amino acids for the assembly of libraries or collections compounds of medical or biological interest.

Using chemical synthetic methodologies to create large diverse libraries of potentially useful compounds permits 25 the synthesis of compounds joined to a solid support of some kind. However, the use of such synthetic methods requires the ability, after synthesis, to identify the structure of the rare members of the library which are able to pass a screening process. Thus, such libraries must be rationally designed so as to permit such identification. This task becomes virtually overwhelming as the number of possible compounds grows multiplicatively.

In attempting to consider this latter point, a number of attempts have been made to devise post-screening methods 30 of "addressing" the specific compounds that the screening process indicates as candidates for further study. One class of such addressable libraries employs a strategy of linking the individual peptides of the library with the nucleic acids encoding them. Examples of such systems, such as the use of biological entities such as bacteriophage displaying the compounds of the library or plasmid-binding proteins fused to member compounds of the library have been described above. However, this methodology is not limited to biological systems, and can be employed by the co-polymerization 35 of the test compound and a corresponding nucleotide sequence onto a single solid support.

Another strategy involves chemically synthesizing the combinatorial libraries on solid supports in a methodical and predetermined fashion, so that the placement of each library member gives information concerning the synthetic structure of that compound. Examples of such methods are described, for example, in Geysen, U.S. Patent No. 4,833,092, in which compounds are synthesized on functionalized polyethylene pins designed to fit a 96 well microtiter dish so 40 that the position of the pin gives the researcher information as to the compound's structure. Similarly Hudson *et al.*, PCT Publication No. WO94/05394, describe methods for the construction of combinatorial libraries of biopolymers, such as polypeptides, oligonucleotides and oligosaccharides, on a spatially addressable solid phase plate coated with a functionalized polymer film. In this system the compounds are synthesized and screened directly on the plate. Knowledge 45 of the position of a given compound on the plate yields information concerning the nature and order of building blocks comprising the compound. Similar methods of constructing addressable combinatorial libraries may be used for the synthesis of compounds other than biopolymers.

Another approach has been the use of large numbers of very small derivatized beads, which are divided into as many equal portions as there are different building blocks. In the first step of the synthesis, each of these portions is reacted with a different building block. The beads are then thoroughly mixed and again divided into the same number 50 of equal portions. In the second step of the synthesis each portion, now theoretically containing equal amounts of each building block linked to a bead, is reacted with a different building block. The beads are again mixed and separated, and the process is repeated as desired to yield a large number of different compounds, with each bead containing only one type of compound.

This methodology, termed the "one-bead one-compound" method, yields a mixture of beads with each bead potentially bearing a different compound. Thus, in this method the beads themselves cannot be considered "addressable" 55 in the same sense as in the solid phase supports and arrays described above, or as in the cellular or phage libraries. However, the compounds displayed in the surface of each bead can be tested for the ability to bind with a specific compound, and, if those (typically) few beads are able to be identified and separated from the other beads, a presumable

pure population of compounds can be recovered and analyzed. Of course, this latter possibility depends upon the ability to load and extract enough information concerning the compounds on the surface of each bead to be susceptible to meaningful subsequent analysis. Such information may simply be in the form of an adequate amount of the compound of interest to be able to determine its structure. For example, in the case of a peptide, enough of the peptide must be synthesized on the bead to be able to perform peptide sequencing and obtain the amino acid sequence of the peptide.

For synthetic chemical libraries, not limited to the one-bead one-compound method, in which the compounds of interest are not naturally-occurring peptides or oligonucleotides, analysis can be a tedious and difficult undertaking. In these cases, a code made from easily synthesized and analyzed "tag" molecules (for example, amino acids or other small multifunctional molecules, such as halogenated aromatics) can be co-synthesized with the compounds comprising the library. After a screening procedure, the tag can be "uncoded" to elucidate the structure of the compounds of interest. The code can be relatively arbitrary, so that the structure of any test compound made of building blocks, in which the building block members are able to be designated as corresponding, for example, to an amino acid (or dipeptide, tripeptide etc.), can be determined in this way.

As described above, the construction of combinatorial libraries provides researchers the opportunity to construct a vast number of potential chemical candidates to answer basic and applied structure-function questions, such as, without limitation: the relationship between a ligand and its receptor, a given antibody and its antigen and an enzyme and substrate. However, the ability to generate large libraries of potential drug compounds overwhelms most available screening methods. Thus, a bottleneck of this emerging and powerful technology remains adequate high-throughput screening procedures to identify the few compounds which are potential candidates for further study from among the thousands, millions or billions of other compounds in the library.

When the combinatorial library is to be screened for the presence of therapeutic or diagnostic agents, candidate compounds are generally initially screened for their ability to bind to a particular member of biological binding partners. By "binding partners" is meant that two or more compounds are able to join under appropriate biological or *in vitro* conditions to form a specific complex. Examples of such binding partners are, without limitation, antibody and antigen, ligand and receptor, and enzyme and substrate. At times, either ligand or receptor, or both may be comprised of a complex of more than one compound or polypeptide chain. For example, in the case of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), the soluble ligand TNF appears to bind to its receptor in the form of a TNF homotrimer; each TNF trimer can bind three copies of the receptor and clustering of the TNF receptor is thought to be required for it to exert its biological effects. Each and all polypeptide chains involved in the binding of the TNF trimer to the clustered receptors are considered individual binding partners.

One common screening method currently applied consists of coating a solid support, such as the wells of a microtiter dish, with the specific molecule for which a binding partner is sought. The library member compounds are then labeled, plated onto the solid support, and allowed to bind the library members. After a wash step, the binding partner complexes are then detected by detection of the label joined to the bound library members. This type of procedure is particularly well suited to combinatorial libraries wherein the member compounds are provided in a solution or medium. This method can be somewhat labor intensive and, in order to achieve the high throughput required to screen such large numbers of test compounds, may as a first step require screening pools of test compounds, followed by one or more rescreening step in order to specifically identify the compound of interest. The situation can also be reversed, so that the library members are allowed to coat individual wells and are probed with the specific molecule.

In cases wherein the combinatorial library is to contain antibody analogs or peptides targeted to a given epitope, the library members may contain a portion of an antibody recognized by a secondary antibody able to be detected, for example in an enzyme-linked immunological assay (ELISA) or by virtue of being directly or indirectly labeled, for example with a radionuclide, a chemiluminescent compound, a fluor, and enzyme or dye.

Tawfik *et al.*, 90 Proc. Natl. Acad. Sci. 373-77 (1993) describe a method of screening a library of antibodies (in this case, from a hybridoma library generated using a mimic of the transition state intermediate of an enzymatic reaction) for the presence of rare antibodies having a desired catalytic activity. The screening compound, in this case the enzyme substrate, was immobilized on 96 well microtiter dishes. Supernatants from each clone were placed into separate wells under conditions promoting the enzymatic reaction. The products of the enzymatic reaction, still immobilized to the microtiter dish, were assayed by the use of product-specific monoclonal antibodies. Again, this type of screening process is quite labor-intensive and may necessitate repetitive screening of pools of test compounds in order to achieve high throughput of large libraries.

In the cellular or phage display libraries and "one-bead one-compound" synthetic libraries described above the library members can be screened for the ability to bind a specific binding partner (*e.g.*, a receptor) which is labeled with a detectable fluor, such as fluorescein or phycoerythrin. Because each particle (for example, a cell or a bead) displays only one species of test compound, the fluorescently labeled particles can be detected and sorted using a fluorescence activated cell sorter (FACS). An enriched population of positive beads or particles can then be rescreened, if necessary, and individually analyzed. This strategy can be employed using cells displaying the test compounds or beads on which the test compounds are synthesized. However, this method also suffers from a lack of ease of use,

and is time intensive.

Whether screening is by the panning procedure previously described or by binding of labels to the solid phase bound test compounds, a common screening procedure is by competitive binding of the test compounds in the presence of a detectable control ligand, often the natural ligand for the specific binding partner to which the test compounds are intended to be directed. Again, this method can be quite labor-intensive and requires the generation of a standard curve and correlation of the data obtained from the competition experiments with the standard curve in order to generate meaningful data. Thus, competition assays are unable to yield easily interpreted and rapid results in an initial screen of thousands or millions of different library members.

ELISA and similar assay formats are useful when the library members are derivatives of antibodies and contain variable regions directed against known antigens. However, these methods may not be as useful in a non-competitive (*i.e.*, direct) format where neither the specific binding partner nor the desired test compounds are antibodies or contain an available epitope against which a secondary antibody can be easily generated.

Biochemical tools have been generated consisting of chimeric peptides containing portions of a peptide ligand and specific domains of an antibody. Such agents have been devised mainly as therapeutic aids to the delivery of drugs within a patient's body. Especially in the case of peptide drugs, such as soluble agonists of cytokines and other such agents, therapeutic agents or drugs often have a short systemic half-life which reduces the stability of such drugs *in vivo*. This reduced stability may, in some cases, be counteracted by higher or more frequent dosages, but this may lead to such undesirable consequences as drug tolerance, toxic effects, and high cost of the drug to the patient.

One strategy for overcoming these shortcomings, particularly with regard to the use of systemic biochemical agonists, has been the use of fusion peptides, which have a longer half life in the circulatory system. These fusion peptides generally contain a binding partner, such as a cytokine receptor, fused to part of an immunoglobulin chain. The immunoglobulin chain acts as molecular camouflage, reducing the opportunity for the binding partner to be recognized as a "foreign" antigen by the organism.

Thus, Shin, *et al.*, 92 *Proc Nat'l Acad. Sci.* 2820-24 (1995) employed fusion peptides made by constructing recombinant vectors having the gene encoding human transferrin fused, in frame, to the 3' end of a chimeric mouse-human IgG3 gene encoding variable and constant regions. The resulting fusion molecules were able to bind antigen (dansyl) and the purified transferrin receptor, and were able to enter the brain parenchyma of rats using the transferrin receptor for transport from the circulatory system. The remaining variable region of the antibody could contain other optional specificities, thus the site is available for secondary targeting of the molecule, such as for therapeutic purposes, once across the blood-brain barrier.

Evans and coworkers, 180 *J. Exp. Med.* 2173-79 (1994), using molecular cloning techniques, reported the construction of a fusion protein containing extracellular portions of the p75 high affinity receptor or, alternatively the p55 low affinity receptor, specific for tissue necrosis factor alpha (TNF $\alpha$ -R) fused to a constant region of human IgG. The soluble, non-fusion forms of the TNF receptors are known to be rapidly degraded *in vivo*. Cells were transformed with vectors expressing portions of heavy immunoglobulin chain fused to each of TNF receptors. The fusion peptide was more stable than the soluble receptor in serum. Moreover, the fusion peptides were secreted as dimers containing two heavy chains bound by disulfide linkages. The dimers were able to bind the TNF trimers (a naturally-occurring conformation of TNF $\alpha$ ) in two separate areas and thus with higher affinity that is possible when the fusion peptide is in the soluble monomeric form.

Other fusion proteins containing a ligand or receptor and an antibody portion have been used in the search for effective therapeutic agonists to humoral agents. In Fountoulakis *et al.*, 270 *J. Biol. Chem.* 3958-64 (1995) the extracellular domain of the human interferon  $\gamma$  receptor was expressed as a fusion protein with the IgG hinge, C $H$ 2 and C $H$ 3 domains, and was shown to bind interferon, compete for interferon binding to the cell surface receptor of tissue culture cells, and inhibit interferon-mediated antiviral activity. Due to the immunoglobulin portion of the fusion protein, the protein was expressed in Chinese Hamster ovary cells as a disulfide-linked homodimer. The dimer was able to bind interferon more strongly than the soluble receptor monomer.

In Pitti, *et al.*, 31 *Molec. Immunol.* 1345-51 (1994) the human interleukin-1 (IL-1) receptor was expressed in transfected human cells as a fusion protein containing the hinge and Fc regions of the IgG heavy chain. This fusion peptide was reported to have an extended pharmacological half-life in the circulatory system of mice and to bind IL-1.

Crowe *et al.*, 168 *J. Immunol. Meth.* 79-89 (1994) expressed a gene containing coding sequences of the extracellular domain of the human lymphotoxin  $\alpha$  receptor fused to a gene segment encoding the constant portion of human IgG heavy chain. The fusion protein was cloned into a baculovirus vector and expressed in both insect cells and African green monkey kidney cells as a dimer. The IgG portion of the fusion peptide was used as a ligand for affinity purification of the fusion peptide, and also enabled disulfide facilitated dimerization of the fusion peptides to provide a high-affinity ligand for lymphotoxin.

These latter five references are incorporated by reference herein.

Summary of the Invention

The present invention is directed to a method of screening candidate biologically active molecules, preferably, though not necessarily contained in combinatorial chemical libraries, in which a multifunctional chimeric protein is constructed and used to directly bind candidate compounds in a screening process for biological activity or binding avidity. The chimeric protein contains at least a portion of a specific binding partner or a peptide analog thereof, with which test compounds are sought to interact. Preferably, the specific binding partner is a ligand or ligand receptor. The chimeric protein also contains at least one portion of an antibody chain which is able to recognize an antigen, able to be recognized as an epitope, and/or which functions as an immunoglobulin hinge domain. In a particularly preferred embodiment the chimeric protein contains an immunoglobulin domain which is able to recognize an antigen and/or able to be recognized as an epitope and also contains the flexible "hinge" region of the immunoglobulin heavy chain placed at a location between the immunoglobulin portion of the chimeric protein and the receptor moiety. Preferably, the immunoglobulin portion of the chimeric protein is derived from an immunoglobulin heavy chain.

15 Detailed Description of the InventionDefinitions:

By "specific molecule" is meant a molecule such as, without limitation, a ligand; a receptor, such as a cell surface receptor able to bind a ligand; an antibody; an antigen; an enzyme; a hormone; and an enzyme substrate. As will be clear from the specification, the chimeric protein used in the methods of the present invention need not contain all of a specific molecule or its peptide analog, but need only contain enough of a portion to be recognized and bound by a given compound. A specific molecule need not be naturally occurring; it only need be a molecule for whom one or more binding partner is sought to be found.

By "peptide analog" is meant a molecule and resembles, with regard to its binding ability and/or specificity, a specific molecule, as defined above. Such peptide analogs may be found or constructed by protein engineering techniques, such methods being well known to those of skill in the art. Alternatively, such peptide analogs may be found by a reiterative screening process, for example wherein a natural binding partner of the specific molecule (which specific molecule is not necessarily a protein or peptide), or a portion thereof, is used as described herein (i.e. in a chimeric protein) to screen peptide compounds for the ability to bind to it. In a second screening step, the newly found peptide compound (or a portion thereof) may itself be used as a peptide analog of the specific molecule in a chimeric protein to screen for analogs of the natural binding partner. Other methods for finding or making peptide analogs will be apparent to those of skill in the art.

By "epitope" is meant an antigen or portion thereof which is capable of binding with an antibody as an antigenic determinant.

By "binding partner complex" is meant the association of two or more molecules which are bound to each other in a specific, detectable manner; thus the association of ligand and receptor, antibody and antigen, and chimeric protein and the compound to which it binds.

By "chimeric protein" is meant a non naturally-occurring protein or polypeptide comprising some or all of the amino acid sequences from at least two different proteins or polypeptides, or of one protein or polypeptide and a non naturally occurring polypeptide chain. As used herein, a chimeric protein is designed, made, or selected intentionally, and contains at least two domains.

By "directly or indirectly labeled" is meant that a molecule may contain a label moiety which moiety emits a signal which is capable of being detected, such as a radioisotope, a dye, or a fluorescent or chemiluminescent moiety, or may contain a moiety, such as an attached enzyme, ligand such as biotin, enzyme substrate, epitope, or nucleotide sequence which is not itself detected but which, through some additional reaction, is capable of indicating the presence of the compound.

By "secondary molecule" is meant a molecule which is able to bind to a region within the second domain of the chimeric protein, thereby allowing its detection or purification.

By "hinge region" or "immunoglobulin heavy chain hinge region" is meant one of a family of proline and cysteine-containing amino acid sequence regions which occur between the C<sub>H</sub>2 and C<sub>H</sub>1 regions of many immunoglobulin heavy chains, or analogs of these amino acid sequences based thereon, in which the regions to the amino and carboxy terminal side of the hinge are spatially separated by a turn or kink in the polypeptide chain so as to facilitate their separate and simultaneous specific binding with other molecules.

By "ligand" is meant a molecule or a multimeric molecular complex which is able to specifically bind another given molecule or molecular complex. Often, though not necessarily, a ligand is soluble while its target is immobilized, such as by an anchor domain imbedded into a cell membrane.

By "receptor" is meant at least a portion of a molecule, or a multimeric molecular complex which has an anchor

domain embedded into a cell membrane and is able to bind a given molecule or molecular complex. Many receptors have particularly high affinity for a ligand when either or both the receptor or ligand are in a homo- or heteromultimeric form, such as a dimer.

5 By "solid support" is meant an insoluble matrix either biological in nature, such as, without limitation, a cell or bacteriophage particle, or synthetic, such as, without limitation, an acrylamide derivative, cellulose, nylon, silica, and magnetized particles, to which soluble molecules may be linked or joined.

By "naturally-occurring" is meant normally found in nature. Although a chemical entity may be naturally occurring in general, it need not be made or derived from natural sources in any specific instance.

10 By "non naturally-occurring" is meant rarely or never found in nature and/or made using organic synthetic methods.

By "bivalent" is meant able to specifically bind two chemical compounds.

By "multivalent" is meant able to specifically bind two or more chemical compounds.

By "bifunctional" means a compound having two distinct chemical groups capable of separate reaction with one or more additional compound.

15 By "multifunctional" is meant a compound having two or more distinct chemical groups capable of separate reaction with one or more additional compound.

By "multimeric complex" is meant the stable covalent or non-covalent association of two or more identical or different polypeptide chains to form a structure capable of recognition by a binding partner.

By "modified" is meant non naturally-occurring or altered in a way that deviates from naturally-occurring compounds.

20 The chimeric protein of the instant invention is useful as a tool in screening a population of compounds for the ability to bind a specific binding partner, at least a portion of said specific binding partner, or a protein or peptide analog thereof, which is comprised in a first binding domain of the chimeric protein. In preferred embodiments the same chimeric molecule also contains a second binding domain comprising at least one immunologically active region (antigenic or antigen-binding) which confers one or more additional binding specificity. This additional specificity may be used as

25 a means for detecting the chimeric protein; for example and without limitation, through the use of a directly or indirectly labeled secondary antibody, or as means for the binding and/or affinity purification of the chimeric protein or compound of interest using, for example, immobilized Protein A or Protein G or an immobilized antibody able to bind the second domain of the chimeric protein. If the second binding domain of the chimeric protein is not derived from an immunoglobulin chain, it may simply comprise a chain of amino acids to which is bound a ligand such as avidin or biotin; however,

30 in such a case the chimeric protein will contain at least a proline-containing hinge region derived from an immunoglobulin chain.

While the method of the present invention is particularly useful as a tool for the screening of combinatorial library members, it may be used to screen bacterial or phage lysates, or in any diagnostic or analytical assay or preparative protocol in which a specific interaction between binding partners is sought to be detected or a compound is sought to be isolated.

35 Examples of biochemicals known or thought to exert biological effects by way of specific or semispecific binding to a receptor or binding partner include the following: growth hormone, human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin-B chain, proinsulin, relaxin A-chain, leptin receptor, fibroblast growth factor, relaxin B-chain, prorelaxin, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hor-

40 mone, glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, tissue plasminogen activator, bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor alpha, tumor necrosis factor beta, enkephalinase human serum albumin, mullerian-inhibiting substance, gonadotropin-

45 associated peptide,  $\beta$  lactamase, tissue factor protein, inhibitin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- $\beta$ , platelet growth factor, transforming growth factor, TGF- $\alpha$ , TGF- $\beta$ , insulin-like growth factor I and II, insulin growth factor binding proteins, CD4, CD8, Dnase, Rnase, latency associated peptide, erythropoietin, osteoinductive factors, interferon -alpha, -beta and -gamma, colony stimulating factors, M-CSF, GM-CSF, G-CSF, stem cell factor, interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, superoxide dismutase, viral antigens, HIV envelope proteins, gp120, gp140, immunoglobulins, and proteins encoded by the Ig supergene family. These proteins, their ligands or receptors, and fragments or portions of

50 these are included as among potential binding partners contained in the first domain of the chimeric protein.

Thus, in one aspect, the present invention is directed to methods for detecting or isolating a compound comprising contacting the compound with a chimeric protein which contains a first domain comprising a specific binding partner, such as at least a portion of a receptor, antigen, antibody, ligand, enzyme, enzyme substrate or other protein as mentioned above, and a second domain comprising at least one region of an immunoglobulin molecule which is able to specifically bind with an antigen or an antibody, wherein the molecule recognized by the first domain is different than the molecule recognized by the second domain. Preferably, the first domain and the second domain are separated by the proline-containing "hinge" region of an immunoglobulin heavy chain so as to sterically separate the two domains.

ecule containing a nucleotide sequence encoding the chimeric protein. The first domain may be situated either to the amino terminal side or the carboxy terminal side of the second domain; in a particularly preferred embodiment the chimeric protein has the first domain situated to the amino terminal side of the second domain.

In this aspect of the invention the compound of interest, if present, will bind to a region within the first domain of the chimeric protein. If the compound is immobilized, such as in a cellular or phage display library or in the "one-bead, one-compound" libraries, the solid support can then be washed free of excess chimeric protein and the chimeric protein: compound conjugate (binding partner complex) detected. In a preferred embodiment, the chimeric protein is detected by binding the second domain of the chimeric protein with a labeled secondary binding partner, such as a enzyme-labeled anti-IgG secondary antibody, specific for a region of the second domain. Detection of the secondary antibody permits identification of solid supports containing compounds which are able to interact with the binding partner of the first domain. These compounds can then be analyzed for elucidation of their structure or in additional assay protocols.

In this preferred embodiment, if the labeled secondary binding partner used to bind the second domain has a fluorescent or pigmented label or contains a moiety that participates in a reaction to form a fluorescent or pigmented product, the candidate compounds linked to solid supports can be separated from non-candidate (*i.e.*, non-binding) compounds using a cell sorter; such instruments, such as fluorescent-activated cell sorters (FACS), are well known in the art. After sorting, individual solid supports can be isolated, the chimeric protein eluted from the bound compound of interest, and the compound characterized. Alternatively, for solid supports containing a tag identifying the immobilized compound, the tag may be "read" to obtain information about the compound. Solid supports may also be sorted by hand, provided the particle is large enough to be so manipulated.

The secondary binding partner may alternatively be joined to a solid support, such as a magnetic sphere to facilitate purification of the binding parner complex. In such a case, application of a magnetic field will allow the beads to be washed free of unbound compounds prior to isolation and purification. Such a strategy may be employed even when the library members are themselves bound to a solid support.

In another aspect, the chimeric protein may be immobilized on a solid support in such a way as to allow binding of the binding partner of the first domain with a compound in solution. Immobilization may be performed by formation of an antibody:antigen binding complex partner between the solid support (*e.g.*, with an anti-IgG antibody covalently joined thereto, or through use of Protein G or Protein A) and the variable region or antigenic epitope of the second domain of the chimeric protein. After contacting the immobilized chimeric protein with a sample suspected of containing one or more compound of interest, other components of the sample may be washed away and the compound(s) then eluted to produce an enriched population of candidate compounds.

In yet another aspect, the present invention is directed to diagnostic assay methods for the detection or quantification of a member of a binding pair, for example, a receptor, cytokine, enzyme, antibody, ligand or the like, in a sample. The method includes contacting a chimeric protein, as described above, with a sample suspected of containing the compound of interest under conditions permitting the binding of the first domain of the chimeric protein and the compound. Preferably, the compound is immobilized on a solid support so that a chimeric protein: compound binding partner complex is formed after said contacting step. The solid support-bound binding complex can then be washed and the complex detected by interaction of the second domain of the chimeric protein with a directly or indirectly labeled ligand, such as a secondary antibody.

In yet another aspect, the invention is directed to methods for rapidly screening members of a chemical combinatorial library. The library members may be contained in solution or may be immobilized on solid phase supports, whether synthetic or biological. The compounds to be screened may be peptides, oligonucleotides, saccharides, mixtures or analogs of any of these molecular types, other organic molecules, or non-organic compounds which are desired to be preliminarily screened on the basis of their interaction with a binding partner. The relationship between the binding partner and the compound to be screened may be, for example, antibody:antigen, ligand: receptor, enzyme:substrate or any other specific binding interaction between a protein binding partner and a compound. It will be understood that such methods may be used to screen and aid in the identification of analogs and non-naturally-occurring mimics or variants of the natural ligands of these binding partners. Additionally, the specific binding partner contained in the chimeric protein need not be a natural ligand but may itself be an analog of a naturally-occurring ligand.

In this aspect of the invention, the members of the combinatorial library are contacted with the chimeric protein under conditions favoring the binding of the binding partner contained in the first domain of the chimeric protein with a ligand. It is preferred that the chimeric protein be joined to at least another chimeric protein, either identical or different, to form a multimer, most preferably a dimer, joined together, for example, one or more disulfide linkage. In this form, the chimeric protein is at least bivalent with respect to the specific binding partner of the first domain and therefore may have the potential to bind a given compound at more than one location, and more strongly than the monomeric form or which the solid support containing monomeric compounds closely packed on the surface of the support. This is particularly true when the compound itself is in multimeric form. Use of chimeric proteins in multimeric form can be of particular advantage in detecting the presence of low- or medium-affinity candidate compounds from within the library; these compounds may have a completely different structure than the high affinity compounds, and elucidation

of alternative ligand structures may yield information valuable in the later design of diverse higher affinity ligands with different chemical, biochemical or physical characteristics.

The chimeric protein can then be used to isolate or detect the library members to which it has bound through a second domain of the chimeric protein comprising at least one region of an immunoglobulin molecule which is able to specifically bind with an antigen or an antibody, wherein the molecule recognized by the first domain is different than the molecule recognized by the second domain. If the members of the combinatorial library are joined to a solid support, the solid support can be washed free of any unbound chimeric protein and the second domain of the specifically bound chimeric protein molecules allowed to bind with a labeled binding partner, such as a fluorescently, enzyme-labeled radioactively, or dye-labeled secondary antibody. Subsequent detection of the label-associated solid support particles permits identification and isolation of the compound of interest.

It will be apparent in light of the instant disclosure, that, if the compounds being screened are peptides, a chimeric protein can be made having a first domain including a known peptide, for example, the extracellular portion of a cell surface receptor for a specific humoral factor. If analogs to the cell surface receptor are desired, one may employ the methods disclosed herein to isolate compounds from a peptide combinatorial library able to bind the receptor. Upon determination of the structure of such a compound, this new compound can be made the "binding partner" portion of the first domain of a new chimeric protein, and the new chimeric protein used to screen the same or a different combinatorial library for analogs of the receptor. It will also be apparent that this method may be employed to obtain "binding analogs" of a given compound even when the structure of the natural binding partner for a given compound is not known.

Thus, another aspect of the present invention is a method of making a chimeric protein useful in the screening of compounds for their ability to bind a given peptide, comprising the construction of a recombinant plasmid containing a nucleotide sequence encoding at least one constant (C) or variable (V) region of an immunoglobulin chain positioned downstream from a promoter sequence. While it is preferred that the portion of the gene encoding the immunoglobulin chain correspond to either the amino terminal region or the carboxy terminal region of the mature immunoglobulin molecule, all that is necessary is that the nucleotide sequence encode a portion of at least one C or V region recognizable by an antigen or antibody. The portion of the nucleotide sequence encoding the immunoglobulin (C) and/or (V) region have a region at either its 3' or 5' end one or more restriction endonuclease sites for insertion of a DNA fragment within the coding sequence. Preferably, the region contains a restriction cluster of about four or more different restriction endonuclease cleavage sequences for facile cloning. If this restriction cluster is located at the 5' side of the immunoglobulin sequences, the restriction cluster must be positioned between the immunoglobulin sequences and the promoter sequence. Also, the cloned immunoglobulin chain portion preferably contains the nucleotide sequence encoding the "hinge" region of an immunoglobulin chain; such a region usually comprises a proline-containing region having at least one cysteine residue. It will be understood that reference to the 3' or 5' side of a particular nucleotide sequence or sequence region refers to the coding strand of the DNA molecule unless indicated otherwise herein. Preferably, the immunoglobulin chain contains sequences derived from an immunoglobulin heavy (H) chain which include constant (C) region nucleotide sequences.

Such a vector can be regarded as a "cassette holder"; that is this portion of the vector is capable of receiving many interchangeable nucleic acid fragments ("cassettes") encoding portions of receptors, ligands, or other binding partners. The fragments should be engineered or selected to contain restriction sites matching those at one end of the immunoglobulin sequences; in such a case, ligating the binding partner fragment into the vector is trivial. Care must be taken, however, to ensure that the binding partner gene fragment ("cassette") is placed in the same reading frame as the immunoglobulin portion of the chimeric gene. This can be accomplished, if necessary through the construction and use of appropriate oligonucleotide primers or linkers containing a number of bases sufficient to place the cassette in the same reading frame as the immunoglobulin portion of the chimeric gene. If desired, one or more of the primers or linkers may also be constructed to incorporate nucleotide sequences comprising one or more restriction endonuclease cleavage site for facile cloning and interchange of subunits of the binding partner.

Suitable cassettes can be easily constructed; as an example by using PCR or another nucleic acid amplification method. Such methods generally utilize at least two primers directed to different strands and to different locations 5' and 3' (with respect to the coding strand) of the gene portion to be cloned. When the gene fragment, encoding, for example, a portion of a receptor molecule is to be cloned at the 5' end of the gene, the 5' portion of the nucleic acid to be amplified will generally contain an ATG start codon. An example of such a primer is shown in the Examples below. Such a primer can also be directed to the untranslated region of the gene 5' of the ATG to be amplified, in order to ensure that other transcription or translation regulatory sequences (such as the TATA box or a ribosomal binding sequence (RBS)) are also included in the amplified nucleic acid. An example of a consensus eukaryotic RBS is: SEQ ID NO: 19; 5'-GCCRCCATGG-3', where "R" is either A or G. The primer may be directed to sequences to the 5' side of such regulatory sequences, may be directed to some or all of such sequences themselves, or may not be designed to amplify such sequences at all. Those of skill in the art will, in light of this disclosure, recognize that for a given binding partner one of these options may optimize the expression of the chimeric gene; determination of which of these three options may be optimal is a matter of routine screening easily performed by those of skill in the art.

The recombinant vector is preferably capable of replication and expression of the chimeric protein in eukaryotic cells; thus the vector will preferably contain an origin of replication allowing the episomal replication in such cells. In such a case, the promoter directly upstream from the cloned synthetic gene encoding the chimeric protein will be one capable of directing transcription in a eukaryotic host. It is also preferable that the vector and host cell be chosen so as to allow the vector to be replicated and transcribed at high copy number by the eukaryotic cell.

5 Expression of such chimeric proteins in eukaryotic cells allows the cell to treat the expressed chimeric protein much like an immunoglobulin molecule. Thus, the chimeric protein may be glycosylated, permitted to form dimers or other multimeric forms and transported to the cell surface for secretion just as a native immunoglobulin would. This also allows the chimeric protein to be harvested from the tissue culture supernatant without lysing the cells, therefore 10 facilitating purification. As described below, Applicant has demonstrated the feasibility of this approach by cloning and expressing the chimeric protein as a secreted product in African green monkey cells.

Purification of the chimeric protein can be performed by exploiting one of the two specific binding domains of the 15 chimeric protein in a minimum of steps by affinity chromatography; for example, by a lized anti-IgG antibody. The chimeric protein can then be eluted from the affinity matrix for use. Alternatively, the cell-free tissue culture medium containing the chimeric protein can be used without further purification.

In embodiments of the invention employing non-biological solid supports, these solid supports are any insoluble or semisoluble matrix on which chemical compounds, including antibodies and other proteins and members of a combinatorial library, can be joined. Such matrices include: nitrocellulose; cellulose derivatives; nylon; controlled pore glass; polystyrene or polyacrylamide derivatives; dendromeres, magnetic beads; particles or microspheres.

20 Additional embodiments of the present invention are directed to methods of using the chimeric proteins described herein. One such method of use - that of utilizing the first domain of the chimeric protein to bind solid supports displaying a compound or library member of interest, identifying the bound chimeric protein by directing a labeled ligand to the second domain of the protein, detecting the label, and sorting the identified solid supports - has been described above. The chimeric protein may also be used in an application in which the candidate compounds are coated onto a microtiter 25 well, the chimeric protein added, and a directly or indirectly labeled ligand directed to the second chimeric protein domain used to identify the bound chimeric protein. An example of indirectly labeled ligands are antibodies labeled with an enzyme, such as horseradish peroxidase or alkaline phosphatase, which can then be exposed to a substrate in a colorimetric reaction to indicate the presence of the compound of interest. The converse of this scheme may also be employed in which the chimeric protein is immobilized and the library members are used to bind thereto. In the 30 interests of increased assay throughput, an initial screen can be performed using mixtures of different compounds, and subsequent screens can then identify the specific compounds of interest.

Additional embodiments can be found in the examples and in the claims which conclude this specification.

#### Examples

##### Example 1: Vector Construction

The commercially available vector pcDNA3 was purchased from Invitrogen Corp., San Diego CA. This eukaryotic/prokaryotic shuttle vector, which is 5.4 kb in length, includes the following elements: the cytomegalovirus (CMV) eukaryotic promoter and the T7 bacteriophage promoter, both promoting transcription in the clockwise direction; the SP6 bacteriophage promoter, promoting transcription in the opposite direction; a polylinker containing restriction sites for, in order from 5' to 3' with respect to the cloned sequences described below: Hind III, Kpn I, Bam H1, BstX I, EcoR I, EcoR V, BstX I, Not I, Xhol, Xba I and Apa I; the SV40 eukaryotic origin of replication, the ColE1 bacterial episomal origin of replication, the ampicillin resistance gene, and the neomycin resistance gene.

45 This plasmid was linearized using the restriction enzymes Not I and Xho I, as follows. A 200 µl reaction mixture containing 30 (New England Biolabs), 10 mM Tris HCl (pH 7.9), 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT and 100 µg/ml BSA (bovine serum albumin) was incubated at 37 °C overnight. The DNA fragments were separated on a 1% agarose gel using TBE (89 mM Tris (pH 8.0), 89 mM boric acid, 2 mM EDTA (ethylene diamine tetraacetic acid)). The large linearized DNA fragment was excised from the gel, the gel slice crushed and the DNA extracted by adsorption on glass 50 particles, and purified by precipitation in ethanol. The purified DNA fragment was resuspended in TE (10 mM Tris (pH 7.5, 1 mM EDTA), and the concentration of the purified DNA fragment ascertained by determining the absorbance of the solution at 260 nm in a spectrophotometer. The isolated DNA was stored at -20 °C until use.

Genomic mouse DNA was prepared from a lysate of frozen NIH3T3 cells (a mouse fibroblast cell line. An aliquot of NIH3T3 cells (5x10<sup>5</sup>) were centrifuged at 2500 xg for 4 minutes and washed three times with PBS (phosphate-buffered saline). The cells were resuspended in 100 µl of a hypotonic buffer (50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM MgCl<sub>2</sub>) containing 0.5% (v/v) TWEEN® 20 nonionic surfactant and 10 µg of proteinase K, and incubated at 56 °C for 45 minutes. The crude lysate was then incubated at 95 °C for 10 minutes, and finally stored at 4 °C.

Cloning of the IgG1 Immunoglobulin Fragment

5 The carboxy-terminal mouse DNA sequences encoding the constant region C<sub>H</sub>2, C<sub>H</sub>3 and hinge domains of the murine IgG1 heavy chain were amplified from NIH3T3 genomic DNA using PCR. The following oligonucleotide primers were synthesized to be complementary to corresponding portions of the immunoglobulin gene. The underlined portion of SEQ ID NO. 1 corresponds to a Not I restriction endonuclease cleavage site, and the bolded underlined portion of SEQ ID NO. 2 corresponds to an Xho I restriction endonuclease cleavage site.

10 Sense primer (SEQ ID NO. 1) :

5' -- AGCTTCGAGC GGCCGCCGTG CCCAGGGATT GTGGTTGTAA G-- 3'

15 Antisense primer (SEQ ID NO. 2) :

5' --GATCCTCGAG TCATTTACCA GGAGAGTGGG AGAGGCT-- 3'

20 The PCR reaction was set up by adding the following reagents to a sterile 0.6 ml microfuge tube in the following order: ten microliters of 10X PCR Buffer II (100 mM Tris HCl (pH 8.3), 500 mM KCl), 6 µl of 25 mM MgCl<sub>2</sub>, 2 µl of a 10 mM solution of each dNTP, 2.5 µl of 10 µM mouse IgG1 sense primer (SEQ ID NO. 1), 2.5 µl of 10 µM mouse IgG1 antisense primer (SEQ ID NO. 2), 0.5 µl (2.5 units) of AMPLITAQ® thermostable DNA polymerase (Perkin Elmer Corp.), 66.5 µl ultra pure water, and one wax bead. The reaction mixture was incubated at 70°C until the wax bead melted, 25 then 10 µl of the NIH3T3 lysate was added. The reaction mixture was placed in a Perkin Elmer 480 Thermal Cycler, and the cycler programmed to run 30 cycles under the following conditions: 1 minute at 94 °C, 55 °C for 1 minute, 72 °C for 1.5 minutes, and held at 4 °C until use.

The amplified DNA from the PCR reaction was gel purified by electrophoresis through a 1% agarose gel in TBE. The DNA band corresponding to the amplified DNA was excised from the gel, and eluted in 40 µl of water as above. 30 The purified amplified IgG1 gene fragment was then digested with the restriction enzymes Not I and Xho I as described above. The restriction digest was run on a 1% agarose/TBE gel, the approximately 1 kb fragment was excised from the gel and the DNA eluted from the gel slice in 40 µl of water. The yield was determined by measuring the optical density of the solution at 260 nm on a Beckman DU600 spectrophotometer.

35 The Xho I- and Not I-digested IgG1 PCR product was ligated into the Xho I- and Not I- digested pcDNA3 vector as follows. The ligation reaction was performed in a total volume of 20 µl containing approximately 100 ng pcDNA3 and 100 ng of the IgG1 PCR fragment. This was incubated in 50 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM ATP, 25 µg/mL BSA with 1 unit of DNA ligase at room temperature overnight.

40 A 1 µl aliquot of the ligation mix was used to transform Stratagene Epicurean Coli SURE® Competent Cells (these cells have the genotype: e14-(McrA-) Δ (mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 (Kan) uvrC [F' proAB lacI<sup>q</sup>ZΔM15 Tn10 (Tet<sup>r</sup>)] and are supplied in a transformation buffer). A 50 µl aliquot of thawed cells was placed on ice with 1 µl of the ligation reaction mixture for 30 minutes, followed by a heat shock at 42°C for 45 seconds. 500 µl of Luria broth was added and the cells incubated at 37°C for 1 hour with shaking. The transformants were plated onto LB (Luria broth plates containing 50 µg/mL ampicillin; pcDNA3 carries the β-lactamase gene, which confers resistance to ampicillin whereas untransformed cells do not contain this gene. Representative transformants were used for the preparation of vector DNA by standard "miniprep" procedures, as described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press 2d ed. 1989).

45 Vector DNA was digested with Not I and Xho I and resolved on a 1% agarose/TBE analytical gel to check for the presence of the cloned, PCR-derived mouse IgG1 constant and hinge region. Vector DNA from clones containing Not I/Xho I inserts was purified as described above prior to nucleic acid sequencing.

50 Nucleic acid sequencing was performed using Applied Biosystems' PRISM® Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions. This protocol employs fluorescently-labeled dideoxyribonucleotides as chain terminators for the sequencing reaction, and the results are automatically recorded. The sequencing reaction mixtures were run on a 4% acrylamide denaturing gels containing urea for 10 hours and the entire sequence of the fragment determined. After verification that a clone contained the proper sequence, a large-scale vector preparation was done. The new vector, containing the mouse IgG1 C<sub>H</sub>2, C<sub>H</sub>3, and hinge regions, was termed pcDNA3-IgG1, disclosed herein as SEQ ID NO: 5. It will be recognized that this vector may be used to clone DNA fragments whose 3' end incorporate a Not I restriction endonuclease site.

55 Applicant has also found that a corresponding segment of the IgG2b heavy chain containing the C<sub>H</sub>2, C<sub>H</sub>3, and

hinge regions can be cloned in a similar manner. These IgG2b chimeric proteins may be preferable for certain applications.

Since the primary structure of many immunoglobulins is known, it will be clear to those of skill in the art that a similar strategy may be employed to clone DNA fragments encoding receptors and other peptide binding partners at a position 3' (rather than 5', as above) to the immunoglobulin-encoding portion of the chimeric gene. Upon expression, the result would be a chimeric protein containing the binding partner at its carboxy terminus. This conformation not only would allow the possibility of presenting the binding partner to the test or library compounds in both amino- and carboxy-oriented aspects, but provides the possibility of including a desired variable region of an immunoglobulin chain, for example a monoclonal antibody, as part of the second domain of the chimeric protein. Moreover, if the V<sub>H</sub>, and at least the C<sub>H</sub>2, C<sub>H</sub>3 immunoglobulin regions and the binding partner were included in the chimeric protein, it would be reasonably expected in light of the present disclosure that such a chimeric protein might not only have one specific binding region within the second domain, but may in fact have two.

#### Cloning of tumor necrosis factor receptor (TNF-R) into pcDNA 3-IdG1

The DNA fragment encoding the extracellular portion of the human tumor necrosis factor- $\alpha$  receptor (TNF-R) was obtained from PCR amplification of total RNA cDNA from human peripheral blood mononuclear cells (PBMC). RNA was collected from the PBMCs using standard procedures. The RNA was reverse transcribed in a reaction mixture containing 1  $\mu$ g PBMC whole RNA, 12.5 mM each dNTP, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 5 mM DTT (dithiothreitol), 20 pmoles of a random deoxyribonucleotide hexamer, and 100 units SUPERSCRIPT<sup>®</sup> reverse transcriptase. The reaction mixture was incubated at 42°C for 1 hour, then at 95°C for 5 minutes, and stored at 4°C until use.

PCR reactions of the PBMC cDNA preparation were performed using the following primers.

#### TNF-R sense primer (SEQ ID NO. 3):

5' --GATCGGATCC **ATGGGCCTCT** CCACCGTGCC TGAC -- 3'

#### TNF-R antisense primer (SEQ ID NO. 4):

5' --AGCTTCGAGC GGCCGCTGTG GTGCCTGAGT CCTCAGTGCC-- 3'

The primer having SEQ ID NO: 3 incorporates a ATG start codon (underlined) and a Bam HI site (bolded) into the amplified nucleic acid.

PCR reactions were performed as described previously. The TNF-R PCR product and the pcDNA3-IgG1 were each digested with BamHI and Not I, and the larger DNA fragments of each reaction were gel purified as described above. The purified TNF-R DNA fragment and vector fragment were then ligated together as described above to yield the chimeric protein expression vector pcDNA3-IgG1-TNF-R, disclosed herein as SEQ ID NO: 6, having the TNF-R fragment in the proper orientation. Vector construction was confirmed by diagnostic restriction digestion and nucleic acid sequencing. Large scale vector preparations were made from the transformed E. coli clone.

#### Example 2: Transfection of African green monkey cells with pcDNA3-IgG1-TNF-R, and expression of the chimeric protein.

The host cells chosen to demonstrate expression of the chimeric protein of the present invention were COS-7 African green monkey kidney cells. This cell line can be used for large scale production of heterologous proteins by transfection and expression of a recombinant vector having appropriate regulatory elements, such as pcDNA3-IgG1-TNF-R.

COS-7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 4500 mg/ml D glucose, 584 mg/ml L-glutamine, and 10% fetal bovine serum (FBS). For transformations, cells were seeded at 1-2 x 10<sup>5</sup> cells/ml and incubated at 37°C at 5% CO<sub>2</sub> until 50-70% confluent. By percentage confluent is meant the percentage of the substrate, such as the microtiter dish bottom, that is occupied by cells. The cells were then transfected as follows. For each transfection a solution was made by mixing 20  $\mu$ l LIPOFECTIN<sup>®</sup> (a cationic lipid preparation containing a 1:1 molar ratio of DOTMA (N-[1-(2-, 3-dioleyloxy) propyl]-N,N,N trimethylammonium chloride) and DOPE (dioleyl phosphatidylethanolamine) with 100  $\mu$ l serum-free medium and the siolusion was allowed to stand at roomm temperature for 30

minutes. One to two microliters of the pcDNA3-IgG1-TNF-R solution was also diluted into 100 µl serum-free emdium. The two solutions were combined, mixed gently and incubated at room temperature for 10-15 minutes. Cells were then overlayed with the DNA-LIPOFECTIN® mixture and incubated overnight at 37°C. Trasfection mixture was then removed and replaced with medium. Expression of the pcDNA-IgG1-TNF-R vector was constitutive in the COS-7 cells.

5 The chimeric protein is secreted into the culture media, and can be harvested by decanting or aspirating the cell-free media. Cell-free supernatant was assayed for secretion of the chimeric protein at 48-72 hours following transfection.

10 Example 3: Screening of compounds coated within microtiter wells using an immunoglobulin-binding partner chimeric protein.

15 Following expression of the chimeric protein, the cell-free culture medium was harvested and tested for the presence of the fusion protein. The wells of a plastic microtiter dish were coated with a preparation of TNF $\alpha$  by addition of 2 ng of recombinant TNF $\alpha$  per well in PBS and overnight incubation at 4°C or 2 hours at room temperature. The wells were then washed three times with wash buffer (PBS containing 0.05% (v/v) TWEEN®-20 non-ionic detergent. Following the wash, the wells were blocked to prevent non-specific binding with PBS containing 1% (w/v) BSA and 0.05% TWEEN®-20 non ionic detergent (blocking buffer). The wells were again washed as before. The culture media was serially diluted two-fold 11 times in the blocking buffer and 50 µl of each dilution (and the undiluted media) was added to the coated, blocked wells. A set of uncoated wells also received the diluted cell-free media. Microtiter plates were then incubated for 2 hours at room temperature, then washed three times as before. The presence of the bound chimeric protein was assayed using 100 µl of a 1:5000 dilution of an anti-mouse IgG antibody labeled with horseradish peroxidase (ELISA).

20 Color development was commenced with addition of 100 µl of a commercially obtained chromogenic horseradish peroxidase (HRP) substrate (TMB Color Reagent, Kurkegaard & Perry Laboratories) to each of the microtiter wells. The plates were incubated at room temperature for up to 20 minutes. Color development in this assay system may be terminated by addition of 100 microliters of a stop solution (Kirkegaard & Perry, product code 50-85-05) to each well.

25 The control wells showed no color development. By contrast, the wells in which a TNF/TNF-R complex had been formed showed a distinct blue to purple color formation. The absorbance of each dilution at 450 nm was measured, the absorbance at 650 nm was subtracted, and the results were plotted. The results are shown below.

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| Dilution             | 1:1   | 1:2   | 1:4   | 1:8   | 1:16  | 1:32  | 1:64  | 1:128  | 1:256 | 1:512 | 1:1024 | 1:2048 |
|----------------------|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|--------|--------|
| Transfected medium   | 1.147 | 1.199 | 1.161 | 0.901 | 0.747 | 0.406 | 0.259 | 0.166  | 0.112 | 0.085 | 0.071  | 0.037  |
| Untransfected Medium | 0.101 | 0.028 | 0.028 | 0.053 | 0.037 | 0.055 | 0.053 | 0.0764 | 0.044 | 0.063 | 0.075  | 0.057  |
| No TNF Control       | 0.136 | 0.032 | 0.030 | 0.035 | 0.038 | 0.035 | 0.029 | 0.023  | 0.028 | 0.027 | 0.029  | 0.040  |

The results indicate that neither the control wells containing tissue culture media from untransfected cells, nor the control wells containing the media from transfected cells in the absence of TNF gave an indication of color formation; i.e. specific binding between the chimeric protein and the TNF binding partner. However, the media from cells transfected with the vector encoding the chimeric protein was able to bind to wells coated with TNF, and gave a titration curve indicating the presence of specific target binding.

Example 4: Screening of particle-bound compounds using an immunoglobulin-binding partner chimeric protein.

Recombinant TNF $\alpha$  (obtained from R & D Systems) was immobilized on cyanogen bromide-activated SEPHAROSE® CL 4B agarose beads as follows. A 0.5 ml aliquot of cyanogen bromide-activated SEPHAROSE® 4B was washed with ice-cold 0.1 N HCl. Ten micrograms of TNF $\alpha$  were dissolved in 10  $\mu$ l PBS, then added to 100  $\mu$ l of a solution of 0.1 M HCO<sub>3</sub> and 0.5 M NaCl. This was mixed with 100  $\mu$ l of the washed, activated SEPHAROSE® beads and the suspension incubated at room temperature for 2 hours.

The unreacted cyanogen bromide-activated sites were blocked by the addition of 500  $\mu$ l of 50 mM glycine (pH 8.0) to the TNF-coupled SEPHAROSE® beads. The same amount of the glycine solution was added to 100  $\mu$ l of washed, uncoupled SEPHAROSE® as a negative control.

Potential sites of non-specific binding of protein to the SEPHAROSE® beads was blocked by resuspending and incubating the two bead slurries (TNF and control) in 10 volumes of 1% (w/v) BSA and in TBST (20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% (v/v) TWEEN® 80 non-ionic surfactant) for 15 minutes at room temperature.

Forty microliters of the TNF and control SEPHAROSE® beads were each exposed to 100  $\mu$ l of tissue culture supernatant from either untransfected or the pCDNA3-IgG1-TNF-R transformed COS-7 cells and incubated at room temperature for 1 hour. The beads were then washed with TBST.

Detection of the bound chimeric protein was accomplished through the use of a secondary anti-mouse IgG1 antibody coupled to alkaline phosphatase (AP). The alkaline phosphatase-coupled antibody, and its chromogenic substrate was obtained from a commercially available kit, the PROTOBLOT® II AP System (Promega Corp.), and used in accordance with the manufacturer's directions. A solution of AP-anti-mouse IgG (1mg/ml) was diluted 1:5000 into Tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl). One hundred microliters of this solution was added to the aliquots of SEPHAROSE® beads and incubated at room temperature for 1 hour. The beads were then washed three times in TBS.

Color development was commenced with addition of 100  $\mu$ l WESTERN BLUE® chromogenic AP substrate to each of the aliquots of SEPHAROSE® beads. These were incubated at room temperature for 20 minutes. Color development in this assay system may be terminated by washing the beads with water. Aliquots of each SEPHAROSE® bead mixture were observed under a microscope using a 10 X objective lens. The control beads remained colorless. By contrast, the beads in which a TNF/TNF-R complex had been formed were stained with a distinct blue to purple color.

Example 5: Construction of Additional Fusion Peptides

Using the pCDNA3-IgG1 "cassette holder" and the same strategy employed in the Examples described above, additional individual chimeric proteins were made having, at the amino terminal regions, extracellular ligand-binding portions of the erythropoietin receptor, FAS (a receptor of the Nerve Growth Factor family having properties similar to TNF $\alpha$ -R), the interleukin 4 receptor, and the interleukin 6 receptor. The nucleotide sequences for these receptors was obtained from the GENBANK nucleotide sequence database. The nucleotide sequences of other binding partners can be obtained from published or database sources, or can be obtained by direct peptide sequencing of an isolated protein.

Primers designed to amplify the extracellular portions of the indicated receptors were employed to obtain PCR-amplified, "clonable" double-stranded DNA. As above, sense primers incorporated a BamH1 site just prior to the ATG initiation codon, and antisense primers incorporated a Not 1 restriction site after the termination codon. Primer sets (with the initiation codon of the sense strand underlined) and the amplified DNA sequences (coding strand sequence only) were as follows :

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Erythropoietin Receptor

Sense primer

SEQ ID NO: 7

5' -GATCGGATCCCATGGACCACCTCGGGCGTCCCTC-3'

Antisense primer

SEQ ID NO: 8

5' -AGCTTCGAGCGGCCGCGGGTCCAGGTCGCTAGGCGTCAG-3'

EPO Receptor DNA sequence amplified:

SEQ ID NO: 9

5' -ATGGACCACCTCGGGCGTCCCTCTGGCCCCAGGTGGCTCCCTTGCTCCT  
GCTCGCTGGGCCGCCTGGCGCCCCCGCTAACCTCCCGACCCAAGTCGAGA  
15 GCAAAGCGGCCTTGCTGGCGCCGGGGGCCGAAGAGCTTCTGTGCTCACCGAG  
CGGTTGGAGGACTTGGTGTGTTCTGGGAGGAAGCGGCGAGCGCTGGGTGGGCC  
GGGCAACTACAGCTTCTCCTACCAGCTCGAGGATGAGCCATGGAAGCTGTGCGCC  
20 TGCACCAGGCTCCCACGGCTCGTGGTGCAGCTCGCTTCTGGTGTGCTGCCTACA  
GCCGACACGTCGAGCTCGTGCCTAGAGTTGCGCGTCACAGCAGCCTCCGGCGC  
TCCCGCATATCACCGTGTATCCACATCAATGAAGTAGTGCTCCTAGACGCCCG  
25 TGGGGCTGGTGGCGCGGTGGCTGACGAGAGCGGCCACGTAGTGTGCGCTGGCTC  
CCGCCGCCTGAGACACCCATGACGTCTCACATCGCTACGAGGTGGACGTCTCGGC  
CGGCAACGGCGCAGGGAGCGTACAGAGGGTGGAGATCCTGGAGGGCCGACCGAGT  
GTGTGCTGAGCAACCTGCGGGGCCGACCGCTACACCTCGCCGTCCGCGCGT  
30 ATGGCTGAGCCGAGCTCGCGGCTTCTGGAGCGCCTGGTCGGAGCCTGTGCGCT  
GCTGACGCCTAGCGACCTGGACCC-3'

Interleukin 4 Receptor

Sense primer

SEQ ID NO: 10

5' -GATCGGATCCATGGGTGGCTTGCTCTGGCTC-3'

Antisense primer

SEQ ID NO: 11

5' -AGCTTCGAGCGGCCGCGTGTGCTCGAAGGGCTCCCTGTA-3'

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IL-4 Receptor DNA sequence amplified

SEQ ID NO: 12

5' -ATGGGGTGGCTTGCTCTGGCCTGTTCCCTGTGAGCTGCCTGGTCCTGCT  
GCAGGTGGCAAGCTCTGGAACATGAAGGTCTGCAGGAGCCCACCTGCGTCTCCG  
ACTACATGAGCATCTCTACTTGCAGTGGAAAGATGAATGGTCCCACCAATTGCAGC  
10 ACCGAGCTCCGCCTGTTGTACAGCTGGTTTCTGCTCTCCGAAGCCCACACGTG  
TATCCCTGAGAACAAACGGAGGCCGGGTGCGTGTGCCACCTGCTCATGGATGACG  
TGGTCAGTGCAGATAACTATACACTGGACCTGTGGCTGGCAGCAGCTGCTGTGG  
15 AAGGGCTCCTCAAGCCCAGCGAGCATGTGAAACCCAGGGCCCCAGGAAACCTGAC  
AGTCACACCAATGTCTCCGACACTCTGCTGCTGACCTGGAGCAACCCGTATCCCC  
CTGACAATTACCTGTATAATCATCTCACCTATGCAGTCACATTGGAGTGAAAAC  
20 GACCCGGCAGATTTCAGAATCTATAACGTGACCTACCTAGAACCCCTCCCTCCGCAT  
CGCAGCCAGCACCCCTGAAGTCTGGATTCCCTACAGGGCACGGGTGAGGGCCTGGG  
CTCAGTGCTATAACACCACCTGGAGTGAGTGGAGCCCCAGCACCAAGTGGCACAAC  
25 TCCTACAGGGAGCCCTCGAGCAGCAC-3'

Interleukin 6 Receptor

Sense primer

SEQ ID NO: 13

5' -GATCGAATTCATGCTGGCCGTCGGCTGCGCGCTG-3'

35 Antisense primer

SEQ ID NO: 14

40 5' -AGCTTCGAGCGGCCGCATCTGCAC~~T~~GGAGGCTTGTGCG-3'

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IL-6 Receptor DNA sequence amplifiedSEQ ID NO: 15

5 ATGCTGGCCGTGGCTGGCGCTGCTGGCTGCCCTGCTGGCCGCCGGAGCGGC  
 GCTGGCCCCAAGGCCTGCCCCGAGGTGGCAAGAGGGCTGCTGACAGTC  
 TGCCAGGAGACAGCGTGACTCTGACCTGCCGGGGTAGAGCCGGAAGACAATGCC  
 10 ACTGTTCACTGGGTGCTCAGGAAGCCGGCTGCAGGCTCCCACCCAGCAGATGGC  
 TGGCATGGGAAGGAGGCTGCTGCTGAGGTCGGTGCAGCTCCACGACTCTGGAAACT  
 ATT CATGCTACCGGGCCGGCCGGCCAGCTGGACTGTGCACTGCTGGTGGATGTT  
 15 CCCCCCGAGGAGCCCCAGCTCTCCTGCTTCCGGAAGAGCCCCCTCAGCAATGTTGT  
 TTGTGAGTGGGGTCCTCGGAGCACCCATCCCTGACGACAAAGGCTGTGCTCTTGG  
 TGAGGAAGTTTCAGAACAGTCCGGCGAAGACTTCCAGGAGCCGTGCCAGTATTCC  
 20 CAGGAGTCCCAGAAGTTCTCCTGCCAGTTAGCAGTCCGGAGGGAGACAGCTCTT

25 CTACATAGTGTCCATGTGCGTCGCCAGTAGTGTGGAGCAAGTTCAGCAAAACTC  
 AACCTTTCAGGGTTGGAATCTTGACGCCCTGATCCGCCTGCCAACATCACAGTC  
 ACTGCCGTGGCCAGAAACCCCCGCTGGCTCAGTGTACCTGGCAAGACCCCCACTC  
 CTGGAACATCATTCTACAGACTACGGTTGAGCTCAGATATCGGGCTGAACGGT  
 30 CAAAGACATTACAACATGGATGGTCAAGGACCTCCAGCATCACTGTGTCACTCCAC  
 GACGCCCTGGAGCGGCCTGAGGCACGTGGTGCAGCTTGTGCCAGGAGGGAGTCGG  
 GCAAGGCGAGTGGAGCGAGTGGAGGCCGAGGCCATGGCACGCCCTGGACAGAAT  
 CCAGGAGTCCCTCAGCTGAGAACGAGGTGTCCACCCCCATGCAGGCACTTACTACT  
 35 AATAAAAGACGATGATAATATTCTTCAAGAGATTCTGCAAATGCGACAAGCCTCCC  
 AGTGCAAGAT-3'

40 FASSense primerSEQ ID NO: 16

45 5' -GATCGGATCCATGCTGGCATCTGGACCCCTCCTACC-3'

Antisense primerSEQ ID NO: 17

50 5' -AGCTTCGAGCGGCCGCGTTAGATCTGGATCCTCCTTTGC-3'

FAS DNA sequence amplifiedSEQ ID NO: 18

5 ATGCTGGGCATCTGGACCCCTCCTACCTCTGGTCTTACGTCTGTTGCTAGATTATC  
 GTCCAAAAGTGTAAATGCCAAGTGACTGACATCAACTCCAAGGGATTGGAATTGA  
 GGAAGACTGTTACTACAGTTGAGACTCAGAACTTGGAAAGGCCTGCATCATGATGGC  
 10 CAATTCTGCCATAAGCCCTGTCCTCCAGGTGAAAGGAAAGCTAGGGACTGCACAGT  
 CAATGGGGATGAACCAGACTGCGTGCCTGCCAAGAAGGGAAAGGAGTACACAGACA  
 AAGCCCATTTCCTCCAAATGCAGAAGATGTAGATTGTGTGATGAAGGACATGGC  
 15 TTAGAAGTGGAAATAAACTGCACCCGGACCCAGAAATACCAAGTGCAGATGTAAACC  
 AACATTTTTGTAACTCTACTGTATGTGAAACACTGTGACCCTGCACCAAATGTG  
 AACATGGAATCATCAAGGAATGCACACTCACCAGCAACACCAAGTGCAAAGAGGAA  
 20 GGATCCAGATCTAAC-3'

The amplified DNA fragments and pDNA3-IgG1 vector were both digested with BamH1 and Not I gel purified, as above, and then the amplified fragments ligated into the restriction-digested vector at a position immediately to the 5' side of the coding region for the hinge-IgG portion of the chimeric protein, again as described above. The recombinant vectors were then used to transfet COS-7 cells, as described above. In each case, the chimeric protein was secreted into the extracellular medium and the ability of each bind its intended ligand was verified.

Example 6: Structure of Secreted Chimeric Protein

30 Aliquots of the extracellular medium of individual chimeric proteins were electrophoresed on reducing and non-reducing SDS-PAGE gels, along with molecular weight standards and an anti GM-CSF monoclonal antibody (bivalent) control. The antibody control and the chimeric proteins showed a marked increase in electrophoretic mobility on the reducing gel as compared to the non-reducing gel, indicating that the secreted cheimeric proteins, like the antibody, are produced as disulfide-linked bivalent dimers.

35 The foregoing examples illustrate particularly preferred embodiments of the present invention, which is not to be construed as limited thereby. Further embodiments are contained throughout the specification and in the claims which follow. Applicant intends that the scope of the invention be determined from the embodiments described or suggested by the specification as a whole, and equivalents thereof.

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SEQUENCE LISTING

5

(1) GENERAL INFORMATION

(i) APPLICANT

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- (A) NAME: Chugai Biopharmaceuticals, Inc.
- (B) STREET: 6275 Nancy Ridge Drive
- (C) CITY: San Diego
- 15 (D) STATE: California
- (E) COUNTRY: USA
- (F) POSTAL CODE: 92121

15

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(ii) TITLE OF THE INVENTION: COMPOSITIONS AND  
METHODS FOR SCREENING DRUG LIBRARIES

25

(iii) NUMBER OF SEQUENCES: 19

30

(iv) COMPUTER READABLE FORM:

35

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

40

(v) CURRENT APPLICATION DATA:

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(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

50

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/627151

(B) FILING DATE: 3 April 1996

55

(2) INFORMATION FOR SEQ ID NO:1:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- 10 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGCTTCGAGC GGCCGCCGTG CCCAGGGATT GTGGTTGTAA G

41

20 (2) INFORMATION FOR SEQ ID NO:2:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- 30 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCCTCGAG TCATTTACCA GGAGAGTGGG AGAGGCT

37

40 (2) INFORMATION FOR SEQ ID NO:3:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs

50

55

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

10 GATCGGATCC ATGGGCCTCT CCACCGTGCC TGAC

34

(2) INFORMATION FOR SEQ ID NO:4:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTTCGAGC GGCCGCTGTG GTGCCTGAGT CCTCAGTGCC

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(2) INFORMATION FOR SEO ID NO:5:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6338 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT 400  
CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT 800  
CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG 1200  
CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA 1600  
CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG 2000  
CTGCTTCGCG ATGTACGGGC CAGATATAACG CGTTGACATT 2400  
GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC 2800

|    |  |      |
|----|--|------|
|    | ATTAGTTCAT AGCCCATAA TGGAGTTCCG CGTTACATAA   | 320  |
| 5  | CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC  | 360  |
|    | CCCGCCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT | 400  |
|    | AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC  | 440  |
|    | TATTTACGGT AAACTGCCA CTTGGCAGTA CATCAAGTGT   | 480  |
| 10 | ATCATATGCC AAGTACGCC CCTATTGACG TCAATGACGG   | 520  |
|    | TAAATGGCCC GCCTGGCATT ATGCCAGTA CATGACCTTA   | 560  |
|    | TGGGACTTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA | 600  |
|    | TCGCTATTAC CATGGTGATG CGGTTTGGC AGTACATCAA   | 640  |
| 15 | TGGGCGTGGA TAGCGGTTG ACTCACGGGG ATTTCCAAGT   | 680  |
|    | CTCCACCCCA TTGACGTCAA TGGGAGTTG TTTTGGCACC   | 720  |
|    | AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC  | 760  |
| 20 | CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG  | 800  |
|    | GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA  | 840  |
|    | CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG  | 880  |
|    | GGAGACCCAA GCTGGCTAGC GTTTAAACTT AAGCTTGGTA  | 920  |
| 25 | CCGAGCTCGG ATCCACTAGT CCAGTGTGGT GGAATTCTGC  | 960  |
|    | AGATATCCAG CACAGTGGCG GCCGCCGTGC CCAGGGATTG  | 1000 |
|    | TGGTTGTAAG CCTTGATAT GTACAGGTAA GTCAGTGGCC   | 1040 |
| 30 | TTCACCTGAC CCAGATGCAA CAAGTGGCAA TGGTTGGAGG  | 1080 |
|    | GTGGCCAGGT ATTGACCTAT TTCCACCTTT CTTCTTCATC  | 1120 |
|    | CTTAGTCCCA GAAGTATCAT CTGTCTTCAT CTTCCCCCA   | 1160 |
| 35 | AAGCCCAAGG ATGTGCTCAC CATTACTCTG ACTCCTAAGG  | 1200 |
|    | TCACGTGTGT TGTGGTAGAC ATCAGCAAGG ATGATCCCAG  | 1240 |
|    | GGTCCAGTTC AGCTGGTTG TAGATGATGT GGAGGGTCAC   | 1280 |
|    | ACAGCTCAGA CGCAACCCCG GGAGGAGCAG TTCAACAGCA  | 1320 |
| 40 | CTTTCCGCTC AGTCAGTGAA CTTCCCATCA TGACCCAGGA  | 1360 |
|    | CTGGCTCAAT GGCAAGGAGT TCAAATGCAG GGTCAACAGT  | 1400 |
|    | GCAGCTTTCC CTGCCCCCAT CGAGAAAACC ATCTCCAAAA  | 1440 |
| 45 | CCAAAGGTGA GAGCTGCAGT GTGTGACATA GAAGCTGCAA  | 1480 |
|    | TAGTCAGTCC ATAGACAGAG CTTGGCATAA CAGACCCCTG  | 1520 |
|    | CCCTGTTCGT GACCTCTGTG CTGACCAATC TCTTACCCA   | 1560 |
|    | CCACACAGGCA GACCGAAGGC TCCACAGGTG TACACCATTG | 1600 |
| 50 | CACCTCCCAA GGAGCAGATG GCCAAGGATA AAGTCAGTCT  | 1640 |
|    | GACCGCCATG ATAACAGACT TCTTCCCTGA AGACATTACT  | 1680 |
|    | GTGGAGTGGC AGTGGAAATGG GCAGCCAGCG GAGAACTACA | 1720 |
| 55 | AGAACACTCA GCCCATCATG AACACGAATG GCTCTTACTT  | 1760 |

|    |   |      |
|----|---|------|
|    | CGTCTACAGC AAGCTCAATG TGCAGAAGAG CAACTGGGAG   | 1800 |
| 5  | GCAGGAAATA CTTTCACCTG CTCTGTGTTA CATGAGGGCC   | 1840 |
|    | TACACAACCA CCATACTGAG AAGAGCCTCT CCCACTCTCC   | 1880 |
|    | TGGTAAATGA CTCGAGTCTA GAGGGCCCGT TTAAACCCGC   | 1920 |
|    | TGATCAGCCT CGACTGTGCC TTCTAGTTGC CAGCCATCTG   | 1960 |
| 10 | TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA CCCTGGAAAGG  | 2000 |
|    | TGCCACTCCC ACTGTCCCTT CCTAATAAAA TGAGGAAATT   | 2040 |
|    | GCATCGCATT GTCTGAGTAG GTGTCAATTCT ATTCTGGGGG  | 2080 |
|    | GTGGGGTGGG GCAGGACAGC AAGGGGGAGG ATTGGGAAGA   | 2120 |
| 15 | CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGCT   | 2160 |
|    | TCTGAGGCCG AAAGAACCAAG CTGGGGCTCT AGGGGGTATC  | 2200 |
|    | CCCACGCGCC CTGTAGCGGC GCATTAAGCG CGGGGGTGT    | 2240 |
| 20 | GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC   | 2280 |
|    | CTAGCGCCCG CTCCCTTCGC TTTCTCCCT TCCTTCTCG     | 2320 |
|    | CCACGTTCGC CGGCTTTCCC CGTCAAGCTC TAAATCGGGG   | 2360 |
| 25 | CATCCCTTTA GGGTTCCGAT TTAGTGCTTT AGGGCACCTC   | 2400 |
|    | GACCCCAAAA AACTTGATTA GGGTGATGGT TCACGTAGTG   | 2440 |
|    | GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT   | 2480 |
|    | GGAGTCCACG TTCTTTAATA GTGGACTCTT GTTCCAAACT   | 2520 |
| 30 | GGAACAACAC TCAACCCTAT CTCGGTCTAT TCTTTGATT    | 2560 |
|    | TATAAGGGAT TTTGGGGATT TCGGCCTATT GGTTAAAAAA   | 2600 |
|    | TGAGCTGATT TAACAAAAAT TTAACCGCAA TTAATTCTGT   | 2640 |
| 35 | GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC   | 2680 |
|    | CCCAGGCAGG CAGAAAGTATG CAAAGCATGC ATCTCAATT   | 2720 |
|    | GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA   | 2760 |
|    | GGCAGAAAGTA TGCAAAGCAT GCATCTCAAT TAGTCAGCAA  | 2800 |
| 40 | CCATAGTCCC GCCCTTAACCT CCGCCCATCC CGCCCCCTAAC | 2840 |
|    | TCCGCCAGT TCCGCCATT CTCCGCCCA TGGCTGACTA      | 2880 |
|    | ATTTTTTTA TTTATGCAGA GGCGGAGGCC GCCTCTGCCT    | 2920 |
| 45 | CTGAGCTATT CCAGAAAGTAG TGAGGAGGCT TTTTGGAGG   | 2960 |
|    | CCTAGGCTTT TGCAAAAGC TCCCAGGAGC TTGTATATCC    | 3000 |
|    | ATTTTCGGAT CTGATCAAGA GACAGGATGA GGATCGTTTC   | 3040 |
| 50 | GCATGATTGA ACAAGATGGA TTGCACGCAG GTTCTCCGGC   | 3080 |
|    | CGCTTGGGTG GAGAGGCTAT TCGGCTATGA CTGGGCACAA   | 3120 |
|    | CAGACAATCG GCTGCTCTGA TGCCGCCGTG TTCCGGCTGT   | 3160 |
|    | CAGCGCAGGG GCGCCCGGTT CTTTTGTCA AGACCGACCT    | 3200 |
| 55 | GTCCGGTGCC CTGAATGAAC TGCAGGACGA GGCAGCGCGG   | 3240 |

|    |   |      |
|----|---|------|
|    | CTATCGTGGC TGGCCACGAC GGGCGTCCT TGCGCAGCTG    | 3280 |
| 5  | TGCTCGACGT TGTCACTGAA GCGGGAAAGGG ACTGGCTGCT  | 3320 |
|    | ATTGGGGCAA GTGCCGGGGC AGGATCTCCT GTCATCTCAC   | 3360 |
|    | CTTGCTCCTG CCGAGAAAGT ATCCATCATG GCTGATGCAA   | 3400 |
|    | TGC GGCGGCT GCATACGCTT GATCCGGCTA CCTGCCATT   | 3440 |
| 10 | CGACCACCAA GCGAAACATC GCATCGAGCG AGCACGTACT   | 3480 |
|    | CGGATGGAAG CCGGTCTTGT CGATCAGGAT GATCTGGACG   | 3520 |
|    | AAGAGCATCA GGGGCTCGCG CCAGCCGAAC TGTCGCCAG    | 3560 |
|    | GCTCAAGGCG CGCATGCCCG ACGGCGAGGA TCTCGTCGTG   | 3600 |
| 15 | ACCCATGGCG ATGCCCTGCTT GCCGAATATC ATGGTGGAAA  | 3640 |
|    | ATGGCCGCTT TTCTGGATTG ATCGACTGTG GCCGGCTGGG   | 3680 |
|    | TGTGGCGGAC CGCTATCAGG ACATAGCGTT GGCTACCCGT   | 3720 |
| 20 | GATATTGCTG AAGAGCTTGG CGGCGAATGG GCTGACCGCT   | 3760 |
|    | TCCTCGTGCT TTACGGTATC GCCGCTCCCG ATTCGCAGCG   | 3800 |
|    | CATCGCCTTC TATCGCCTTC TTGACGAGTT CTTCTGAGCG   | 3840 |
| 25 | GGACTCTGGG GTTCGAAATG ACCGACCAAG CGACGCCAA    | 3880 |
|    | CCTGCCATCA CGAGATTTCG ATTCCACCGC CGCCTTCTAT   | 3920 |
|    | GAAAGGTTGG GCTTCGGAAT CGTTTCCGG GACGCCGGCT    | 3960 |
|    | GGATGATCCT CCAGCGCGGG GATCTCATGC TGGAGTTCTT   | 4000 |
| 30 | CGCCCACCCC AACTGTTTA TTGCAGCTTA TAATGGTTAC    | 4040 |
|    | AAATAAAGCA ATAGCATCAC AAATTCACA AATAAAGCAT    | 4080 |
|    | TTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT    | 4120 |
| 35 | CAATGTATCT TATCATGTCT GTATACCGTC GACCTCTAGC   | 4160 |
|    | TAGAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT   | 4200 |
|    | GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC   | 4240 |
| 40 | CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG   | 4280 |
|    | AGCTAACTCA CATTAAATTGC GTTGGCTCA CTGCCCGCTT   | 4320 |
|    | TCCAGTCGGG AAACCTGTCT TGCCAGCTGC ATTAATGAAT   | 4360 |
| 45 | CGGCCAACGC GCGGGGAGAG GCGGTTGCG TATTGGCGC     | 4400 |
|    | TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTG    | 4440 |
|    | TTCCGGCTGCG GCGAGCGGT A TAGCTCACT CAAAGGCGGT  | 4480 |
|    | AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG   | 4520 |
| 50 | AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA   | 4560 |
|    | AAAAGGCCGC GTTGGCTGGCG TTTTCCATA GGCTCCGCC    | 4600 |
|    | CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG   | 4640 |
| 55 | TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCCTTTC   | 4680 |
|    | CCCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCCT | 4720 |

|    |  |      |
|----|--|------|
|    | GCCGCTTACC GGATAACCTGT CCGCCTTCT CCCTTCGGGA  | 4760 |
| 5  | AGCGTGGCGC TTTCTCAATG CTCACGCTGT AGGTATCTCA  | 4800 |
|    | GTTCGGTGTA GGTGCGTTCGC TCCAAGCTGG GCTGTGTGCA | 4840 |
|    | CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT  | 4880 |
|    | AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT  | 4920 |
| 10 | CGCCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC | 4960 |
|    | GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG  | 5000 |
|    | CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT  | 5040 |
|    | GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG  | 5080 |
| 15 | TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT  | 5120 |
|    | GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAAA | 5160 |
|    | AAGGATCTCA AGAAGATCCT TTGATCTTT CTACGGGGTC   | 5200 |
| 20 | TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTG   | 5240 |
|    | GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT  | 5280 |
|    | TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA  | 5320 |
| 25 | TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAACATCGT | 5360 |
|    | GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA  | 5400 |
|    | TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG  | 5440 |
|    | GGAGGGCTTA CCATCTGGCC CCAGTGTGC AATGATAACCG  | 5480 |
| 30 | CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA  | 5520 |
|    | ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC  | 5560 |
|    | AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG  | 5600 |
| 35 | GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCAGCA | 5640 |
|    | ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTACGCGTC  | 5680 |
|    | GTCGTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA   | 5720 |
| 40 | TCAAGGCAG AGTCTATGATC CCCCATGTTG TGCAAAAAAG  | 5760 |
|    | CGGTTAGCTC CTTGGTCCT CCGATCGTTG TCAGAAGTAA   | 5800 |
|    | GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG  | 5840 |
|    | CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT  | 5880 |
| 45 | CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA  | 5920 |
|    | GTGTATGCGG CGACCGAGTT GCTCTTGCCTT GGGGTCAATA | 5960 |
|    | CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC  | 6000 |
| 50 | TCATCATTGG AAAACGTTCT TCAGGGCGAA AACTCTCAAG  | 6040 |
|    | GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT  | 6080 |
|    | CGTGCACCCA ACTGATCTTC AGCATTTTT ACTTTCACCA   | 6120 |
|    | GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC  | 6160 |
| 55 | AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC  | 6200 |

|   |   |      |
|---|---|------|
| 5 | ATACTCTTCC TTTTCATAA TTATTGAAGC ATTTATCAGG  | 6240 |
|   | GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTAA | 6280 |
|   | GAAAAATAAA CAAATAGGGG TTCCCGGCAC ATTTCCCCGA | 6320 |
|   | AAAGTGCCAC CTGACGTC                         | 6338 |

10 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 6926 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

|    |  |     |
|----|--|-----|
| 25 | GACGGATCGG GAGATCTCCC GATCCCTAT GGTCGACTCT   | 40  |
|    | CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT  | 80  |
|    | CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG  | 120 |
|    | CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA  | 160 |
| 30 | CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTGCG   | 200 |
|    | CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT  | 240 |
|    | GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGTC   | 280 |
| 35 | ATTAGTTCAT AGCCCATAA TGGAGTTCCG CGTTACATAA   | 320 |
|    | CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC  | 360 |
|    | CCCGCCCAATT GACGTCAATA ATGACGTATG TTCCCATAGT | 400 |
| 40 | AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC  | 440 |
|    | TATTTACGGT AAACTGCCA CTTGGCAGTA CATCAAGTGT   | 480 |
|    | ATCATATGCC AAGTACGCC CCTATTGACG TCAATGACGG   | 520 |
|    | TAAATGGCCC GCCTGGCATT ATGCCAGTA CATGACCTTA   | 560 |
| 45 | TGGGACTTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA | 600 |
|    | TCGCTATTAC CATGGTGATG CGGTTTGGC AGTACATCAA   | 640 |
|    | TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT  | 680 |
| 50 | CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTGGCACC   | 720 |
|    | AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC  | 760 |
|    | CCCATTGACG CAAATGGGCG GTAGGGCGTGT ACGGTGGGAG | 800 |
| 55 | GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA  | 840 |
|    | CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG  | 880 |

|    |  |      |
|----|--|------|
|    | GGAGACCAA GCTGGCTAGC GTTTAAACTT AAGCTTGGTA   | 920  |
| 5  | CCGAGCTCGG ATCCATGGGC CTCTCCACCG TGCGCTGACCT | 960  |
|    | GCTGCTGCCG CTGGTGTCTCC TGGAGCTGTT GGTGGGAATA | 1000 |
|    | TACCCCTCAG GGGTTATTGG ACTGGTCCCT CACCTAGGGG  | 1040 |
|    | ACAGGGAGAA GAGAGATAGT GTGTGTCCCC AAGGAAAATA  | 1080 |
| 10 | TATCCACCCCT CAAAATAATT CGATTTGCTG TACCAAGTGC | 1120 |
|    | CACAAAGGAA CCTACTTGTA CAATGACTGT CCAGGCCCCGG | 1160 |
|    | GGCAGGATAC GGACTGCAGG GAGTGTGAGA GCGGCTCCTT  | 1200 |
|    | CACCGCTTCA GAAAACCACC TCAGACACTG CCTCAGCTGC  | 1240 |
| 15 | TCCAAATGCC GAAAGGAAAT GGGTCAGGTG GAGATCTCTT  | 1280 |
|    | CTTGCACAGT GGACCGGGAC ACCGTGTGTG GCTGCAGGAA  | 1320 |
|    | GAACCAGTAC CGGCATTATT GGAGTGAAAA CCTTTTCCAG  | 1360 |
| 20 | TGCTTCAATT GCAGCCTCTG CCTCAATGGG ACCGTGCACC  | 1400 |
|    | TCTCCTGCCA GGAGAAACAG AACACCGTGT GCACCTGCCA  | 1440 |
|    | TGCAGGTTTC TTTCTAAGAG AAAACGAGTG TGTCTCCTGT  | 1480 |
| 25 | AGTAACTGTA AGAAAAGCCT GGAGTGCACG AAGTTGTGCC  | 1520 |
|    | TACCCCAGAT TGAGAATGTT AAGGGCACTG AGGACTCAGG  | 1560 |
|    | CACCACAGCG GCCGCCGTGC CCAGGGATTG TGGTTGTAAG  | 1600 |
|    | CCTTGCATAT GTACAGGTAA GTCACTGGCC TTCACCTGAC  | 1640 |
| 30 | CCAGATGCAA CAAGTGGCAA TGGTTGGAGG GTGGCCAGGT  | 1680 |
|    | ATTGACCTAT TTCCACCTTT CTTCTTCATC CTTAGTCCCA  | 1720 |
|    | GAAGTATCAT CTGTCTTCAT CTTCCCCCA AAGCCCAAGG   | 1760 |
| 35 | ATGTGCTCAC CATTACTCTG ACTCCTAACCG TCACGTGTGT | 1800 |
|    | TGTGGTAGAC ATCAGCAAGG ATGATCCCGA GGTCCAGTTC  | 1840 |
|    | AGCTGGTTG TAGATGATGT GGAGGTGCAC ACAGCTCAGA   | 1880 |
|    | CGCAACCCCG GGAGGAGCAG TTCAACAGCA CTTTCCGCTC  | 1920 |
| 40 | AGTCAGTGAA CTTCCCATCA TGCACCAGGA CTGGCTCAAT  | 1960 |
|    | GGCAAGGAGT TCAAATGCAG GGTCAACAGT GCAGCTTCC   | 2000 |
|    | CTGCCCTCAT CGAGAAAACC ATCTCCAAAA CCAAAGGTGA  | 2040 |
| 45 | GAGCTGCAGT GTGTGACATA GAAGCTGCAA TAGTCAGTCC  | 2080 |
|    | ATAGACAGAG CTTGGCATAA CAGACCCCTG CCCTGTTCGT  | 2120 |
|    | GACCTCTGTG CTGACCAATC TCTTACCCA CCCACAGGCA   | 2160 |
| 50 | GACCGAAGGC TCCACAGGTG TACACCATTG CACCTCCCAA  | 2200 |
|    | GGAGCAGATG GCCAAGGATA AAGTCAGTCT GACCGCCATG  | 2240 |
|    | ATAACAGACT TCTTCCCTGA AGACATTACT GTGGAGTGGC  | 2280 |
| 55 | AGTGGAATGG GCAGGCCAGCG GAGAACTACA AGAACACTCA | 2320 |
|    | GCCCCATCATG AACACGAATG GCTCTTACTT CGTCTACAGC | 2360 |

|    |   |      |
|----|---|------|
|    | AAGCTCAATG TGCAGAAAGAG CAACTGGGAG GCAGGAAATA  | 2400 |
| 5  | CTTTCACCTG CTCTGTGTTA CATGAGGGCC TACACAACCA   | 2440 |
|    | CCATACTGAG AAGAGCCTCT CCCACTCTCC TGGTAAATGA   | 2480 |
|    | CTCGAGTCTA GAGGGCCCGT TTAAACCCGC TGATCAGCCT   | 2520 |
|    | CGACTGTGCC TTCTAGTTGC CAGCCATCTG TTGTTTGCCC   | 2560 |
| 10 | CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG TGCCACTCCC   | 2600 |
|    | ACTGTCCCTT CCTAATAAAA TGAGGAAATT GCATCGCATT   | 2640 |
|    | GTCTGAGTAG GTGTCATTCT ATTCTGGGG GTGGGGTGGG    | 2680 |
| 15 | GCAGGACAGC AAGGGGGAGG ATTGGGAAGA CAATAGCAGG   | 2720 |
|    | CATGCTGGGG ATGCGGTGGG CTCTATGGCT TCTGAGGCGG   | 2760 |
|    | AAAGAACCAAG CTGGGGCTCT AGGGGGTATC CCCACGCGCC  | 2800 |
|    | CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG   | 2840 |
| 20 | CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCC   | 2880 |
|    | CTCCTTTCGC TTTCTCCCT TCCTTTCTCG CCACGTTCGC    | 2920 |
|    | CGGCTTTCCC CGTCAAGCTC TAAATCGGGG CATCCCTTTA   | 2960 |
| 25 | GGGTTCCGAT TTAGTGTCTT ACGGCACCTC GACCCCCAAA   | 3000 |
|    | AACTTGATTA GGGTGATGGT TCACGTAGTG GGCCATCGCC   | 3040 |
|    | CTGATAGACG GTTTTCGCC CTTTGACGTT GGAGTCCACG    | 3080 |
|    | TTCTTTAATA GTGGACTCTT GTTCCAAACT GGAACAAACAC  | 3120 |
| 30 | TCAACCCAT CTCGGTCTAT TCTTTGATT TATAAGGGAT     | 3160 |
|    | TTTGGGGATT TCGGCCTATT GGTTAAAAAA TGAGCTGATT   | 3200 |
|    | TAACAAAAAT TTAACGCGAA TTAATTCTGT GGAATGTGTG   | 3240 |
| 35 | TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGGCAGG   | 3280 |
|    | CAGAAGTATG CAAAGCATGC ATCTCAATT A GTCAGCAACC  | 3320 |
|    | AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAAGTA  | 3360 |
|    | TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC   | 3400 |
| 40 | GCCCCCTAACT CCGCCCCATCC CGCCCCCTAAC TCCGCCAGT | 3440 |
|    | TCCGCCATT CTCCGCCCA TGGCTGACTA ATTTTTTTA      | 3480 |
|    | TTTATGCAGA GGCGGAGGCC GCCTCTGCCT CTGAGCTATT   | 3520 |
| 45 | CCAGAAGTAG TGAGGGAGGCT TTTTTGGAGG CCTAGGCTTT  | 3560 |
|    | TGCAAAAAGC TCCCGGGAGC TTGTATATCC ATTTTCGGAT   | 3600 |
|    | CTGATCAAGA GACAGGATGA GGATCGTTTC GCATGATTGA   | 3640 |
| 50 | ACAAGATGGA TTGCACGCAG GTTCTCCGGC CGCTTGGGTG   | 3680 |
|    | GAGAGGCTAT TCGGCTATGA CTGGGCACAA CAGACAATCG   | 3720 |
|    | GCTGCTCTGA TGCCGCCGTG TTCCGGCTGT CAGCGCAGGG   | 3760 |
| 55 | GCGCCCGGTT CTTTTGTCA AGACCGACCT GTCCGGTGCC    | 3800 |
|    | CTGAATGAAC TGCAGGACGA GGCAGCGCGG CTATCGTGGC   | 3840 |

|    |  |      |
|----|--|------|
|    | TGGCCACGAC GGGCGTTCCCT TGCGCAGCTG TGCTCGACGT   | 3880 |
| 5  | TGTCACTGAA GCGGGAAGGG ACTGGCTGCT ATTGGGCGAA    | 3920 |
|    | GTGCCGGGGC AGGATCTCCT GTCATCTCAC CTTGCTCCTG    | 3960 |
|    | CCGAGAAAGT ATCCATCATG GCTGATGCAA TGC GGCGGCT   | 4000 |
|    | GCATAACGCTT GATCCGGCTA CCTGCCATT CGACCACCAA    | 4040 |
| 10 | GCGAACACATC GCATCGAGCG AGCACCGTACT CGGATGGAAG  | 4080 |
|    | CCGGTCTTGT CGATCAGGAT GATCTGGACG AAGAGCATCA    | 4120 |
|    | GGGGCTCGCG CCAGCCGAAC TGTCGCCAG GCTCAAGGCG     | 4160 |
| 15 | CGCATGCCCG ACGGCGAGGA TCTCGTCGTG ACCCATGGCG    | 4200 |
|    | ATGCCTGCTT GCCGAATATC ATGGTGGAAA ATGGCCGCTT    | 4240 |
|    | TTCTGGATTC ATCGACTGTG GCCGGCTGGG TGTGGCGGAC    | 4280 |
|    | CGCTATCAGG ACATAGCGTT GGCTACCCGT GATATTGCTG    | 4320 |
| 20 | AAGAGCTTGG CGGCGAATGG GCTGACCGCT TCCTCGTGCT    | 4360 |
|    | TTACGGTATC GCCGCTCCCG ATTGCGAGCG CATCGCCTTC    | 4400 |
|    | TATCGCCTTC TTGACGAGTT CTTCTGAGCG GGA C T CTGGG | 4440 |
| 25 | GTTCGAAATG ACCGACCAAG CGACGCCAA CCTGCCATCA     | 4480 |
|    | CGAGATTTCG ATTCCACCGC CGCCTTCTAT GAAAGGTTGG    | 4520 |
|    | GCTTCGGAAT CGTTTCCGG GACGCCGGCT GGATGATCCT     | 4560 |
|    | CCAGCGCGGG GATCTCATGC TGGAGTTCTT CGCCCACCCC    | 4600 |
| 30 | AACTTGTTTA TTGCAGCTTA TAATGGTTAC AAATAAAGCA    | 4640 |
|    | ATAGCATCAC AAATTTACA AATAAAGCAT TTTTTCACT      | 4680 |
|    | GCATTCTAGT TGTGGTTGT CCAAACTCAT CAATGTATCT     | 4720 |
| 35 | TATCATGTCT GTATACCGTC GACCTCTAGC TAGAGCTTGG    | 4760 |
|    | CGTAATCATG GTCATAGCTG TTTCTGTGT GAAATTGTTA     | 4800 |
|    | TCCGCTACA ATTCCACACA ACATACGAGC CGGAAGCATA     | 4840 |
|    | AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA    | 4880 |
| 40 | CATTAATTGC GTT GCGCTCA CTGCCCGCTT TCCAGTCGGG   | 4920 |
|    | AAACCTGTCTG TGCCAGCTGC ATTAATGAAT CGGCCAACGC   | 4960 |
|    | GCGGGGAGAG GCGGTTGCG TATTGGGCGC TCTCCGCTT      | 5000 |
| 45 | CCTCGCTCAC TGACTCGCTG CGCTCGGTG TTCGGCTGCG     | 5040 |
|    | GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA    | 5080 |
|    | TCCACAGAAAT CAGGGATAA CGCAGGAAAG AACATGTGAG    | 5120 |
| 50 | CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC    | 5160 |
|    | GTTGCTGGCG TTTTCCATA GGCTCCGCC CCCTGACGAG      | 5200 |
|    | CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC    | 5240 |
| 55 | CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG    | 5280 |
|    | CTCCCTCGTG CGCTCTCCTG TTCCGACCCCT GCCGCTTACC   | 5320 |

|    |  |      |
|----|--|------|
|    | GGATACCTGT CCGCCTTCT CCCTCGGGA AGCGTGGCGC    | 5360 |
| 5  | TTTCTCAATG CTCACGCTGT AGGTATCTCA GTTCGGTGTA  | 5400 |
|    | GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC  | 5440 |
|    | GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC  | 5480 |
| 10 | TTGAGTCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC   | 5520 |
|    | AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA  | 5560 |
|    | GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG  | 5600 |
|    | GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT  | 5640 |
| 15 | GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA  | 5680 |
|    | TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG  | 5720 |
|    | TTTGAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA   | 5760 |
|    | AGAAGATCCT TTGATCTTT CTACGGGGTC TGACGCTCAG   | 5800 |
| 20 | TGGAACGAAA ACTCACGTTA AGGGATTGGT GTCATGAGAT  | 5840 |
|    | TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA  | 5880 |
|    | ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT  | 5920 |
| 25 | TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA  | 5960 |
|    | TCTCAGCGAT CTGCTCTATT CGTTCATCCA TAGTTGCCGTG | 6000 |
|    | ACTCCCCGTC GTGTAGATAA CTACGATAACG GGAGGGCTTA | 6040 |
|    | CCATCTGGCC CCAGTGCTGC AATGATAACCG CGAGACCCAC | 6080 |
| 30 | GCTCACCGGC TCCAGATTAA TCAGCAATAA ACCAGCCAGC  | 6120 |
|    | CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC  | 6160 |
|    | GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG  | 6200 |
| 35 | TAAGTAGTTC GCCAGTTAA AGTTTGCAGCA ACGTTGTTGC  | 6240 |
|    | CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTGGT   | 6280 |
|    | ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCAG   | 6320 |
| 40 | TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC  | 6360 |
|    | CTTCGGTCCT CCGATCGTTG TCAGAAAGTAA GTTGGCCGCA | 6400 |
|    | GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC  | 6440 |
|    | TTACTGTCAT GCCATCCGTA AGATGTTTT CTGTGACTGG   | 6480 |
| 45 | TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG  | 6520 |
|    | CGACCGAGTT GCTCTTGCCTT GGCGTCAATA CGGGATAATA | 6560 |
|    | CCCGGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG  | 6600 |
| 50 | AAAACGTTCT TCAGGGCGAA AACTCTCAAG GATCTTACCG  | 6640 |
|    | CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA  | 6680 |
|    | ACTGATCTTC AGCATCTTT ACTTTCACCA GCGTTCTGG    | 6720 |
|    | GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA  | 6760 |
| 55 | ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC  | 6800 |

|   |   |      |
|---|---|------|
| 5 | TTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT  | 6840 |
|   | CATGAGCGGA TACATATTG AATGTATTTA GAAAAATAAA  | 6880 |
|   | CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC | 6920 |
|   | CTGACG                                      | 6926 |

10 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

|    |                                      |    |
|----|--------------------------------------|----|
| 25 | GATCGGATCC ATGGACCACC TCGGGCGTC CCTC | 34 |
|----|--------------------------------------|----|

(2) INFORMATION FOR SEQ ID NO:8:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

|    |   |    |
|----|---|----|
| 45 | AGCTTCGAGC GGCCGCGGGG TCCAGGTCGC TAGGCGTCAG | 40 |
|----|---|----|

(2) INFORMATION FOR SEQ ID NO:9:

50 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 750 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

|    |  |     |
|----|--|-----|
| 5  | ATGGACCACC TCGGGGCGTC CCTCTGGCCC CAGGTCGGCT  | 40  |
|    | CCCTTTGTCT CCTGCTCGCT GGGGCCGCCT GGGCGCCCCC  | 80  |
|    | GCCTAACCTC CGGGACCCCCA AGTTCGAGAG CAAAGCGGCC | 120 |
| 10 | TTGCTGGCGG CCCGGGGGCC CGAAGAGCTT CTGTGCTTCA  | 160 |
|    | CCGAGCGGTT GGAGGACTTG GTGTGTTCT GGGAGGAAGC   | 200 |
|    | GGCGAGCGCT GGGGTGGGCC CGGGCAACTA CAGCTTCTCC  | 240 |
|    | TACCAAGCTCG AGGATGAGCC ATGGAAGCTG TGTCGCTTGC | 280 |
| 15 | ACCAGGCTCC CACGGCTCGT GGTGCGGTGC GCTTCTGGTG  | 320 |
|    | TTCGCTGCCT ACAGCCGACA CGTCGAGCTT CGTGCCCCTA  | 360 |
|    | GAGTTGCGCG TCACAGCAGC CTCCGGCGCT CCGCGATATC  | 400 |
| 20 | ACCGTGTCTAC CCACATCAAT GAAGTAGTGC TCCTAGACGC | 440 |
|    | CCCCGTGGGG CTGGTGGCGC GGTTGGCTGA CGAGAGCGGC  | 480 |
|    | CACGTAGTGT TGCGCTGGCT CCCGCCGCCT GAGACACCCA  | 520 |
| 25 | TGACGTCTCA CATCCGCTAC GAGGTGGACG TCTCGGCCGG  | 560 |
|    | CAACGGCGCA GGGAGCGTAC AGAGGGTGGA GATCCTGGAG  | 600 |
|    | GGCCGCACCG AGTGTGTGCT GAGCAACCTG CGGGGCCGGA  | 640 |
|    | CGCGCTACAC CTTCGCCGTC CGCGCGCGTA TGGCTGAGCC  | 680 |
| 30 | GAGCTTCGGC GGCTTCTGGA GCGCCTGGTC GGAGCCTGTG  | 720 |
|    | TCGCTGCTGA CGCCTAGCGA CCTGGACCCC             | 750 |

## 35 (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

|    |                                       |    |
|----|---------------------------------------|----|
| 50 | GATCGGATCC ATGGGGTGGC TTTGCTCTGG GCTC | 34 |
|----|---------------------------------------|----|

## (2) INFORMATION FOR SEQ ID NO:11:

55

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTCCGAGC GGCGCGGTGC TGCTCGAAGG GCTCCCTGTA 40

15 (2) INFORMATION FOR SEO ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 696 base pair
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

|    |   |     |
|----|---|-----|
| 30 | ATGGGGTGGC TTTGCTCTGG GCTCCTGTT CCTGTGAGCT<br>GCCTGGTCCT GCTGCAGGTG GCAAGCTCTG GGAACATGAA<br>GGTCTTGCAG GAGCCCACCT GCGTCTCCGA CTACATGAGC    | 40  |
| 35 | ATCTCTACTT GCGAGTGGAA GATGAATGGT CCCACCAATT<br>GCAGCACCGA GCTCCGCCCTG TTGTACCAGC TGTTTTCT<br>GCTCTCCGAA GCCCACACGT GTATCCCTGA GAACAACGGA    | 80  |
| 40 | GGCGCGGGGT GCGTGTGCCA CCTGCTCATG GATGACGTGG<br>TCAGTGCAGGA TAACTATACA CTGGACCTGT GGGCTGGGCA<br>GCAGCTGCTG TGGAAAGGGCT CCTTCAAGCC CAGCGAGCAT | 120 |
| 45 | GTGAAACCCA GGGCCCCAGG AAACCTGACA GTTCACACCA<br>ATGTCTCCGA CACTCTGCTG CTGACCTGGA GCAACCCGTA<br>TCCCCCTGAC AATTACCTGT ATAATCATCT CACCTATGCA   | 160 |
| 50 | GTCAACATTT GGAGTGAAAA CGACCCGGCA GATTTCAGAA<br>TCTATAAACGT GACCTACCTA GAACCCCTCCC TCCGCATCGC<br>AGCCAGCACC CTGAAGTCTG GGATTTCTA CAGGGCACGG  | 200 |
| 55 | GTGAGGGCCT GGGCTCAGTG CTATAACACC ACCTGGAGTG<br>AGTGGAGCCC CAGCACCAAG TGGCACAACT CCTACAGGGA<br>GCCCTTCGAG CAGCAC                             | 240 |
|    |   | 280 |
|    |   | 320 |
|    |   | 360 |
|    |   | 400 |
|    |   | 440 |
|    |   | 480 |
|    |   | 520 |
|    |   | 560 |
|    |   | 600 |
|    |   | 640 |
|    |   | 680 |
|    |   | 696 |

(2) INFORMATION FOR SEQ ID NO:13:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

15 GATCGAATTC ATGCTGGCCG TCGGCTGCGC GCTG

34

20 (2) INFORMATION FOR SEQ ID NO:14:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTTCGAGC GGCCGCATCT TGCAC TGCACTGGGA GGCTTGTGCG

40

35 (2) INFORMATION FOR SEQ ID NO:15:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1074 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

50 ATGCTGGCCG TC GGCTGCGC GCTGCTGGCT GCCCTGCTGG

40

CCGCGCCCCGG AGCGGGCGCTG GCCCCAAGGC GCTGCCCTGC

80

GCAGGAGGTG GCAAGAGGCG TGCTGACCAG TCTGCCAGGA

120

|    |  |      |
|----|--|------|
|    | GACAGCGTGA CTCTGACCTG CCCGGGGTA GAGCCGGAAG   | 160  |
| 5  | ACAATGCCAC TGTTCACTGG GTGCTCAGGA AGCCGGCTGC  | 200  |
|    | AGGCTCCCAC CCCAGCAGAT GGGCTGGCAT GGGAAAGGAGG | 240  |
|    | CTGCTGCTGA GGTGGTGCA GCTCCACGAC TCTGGAAACT   | 280  |
| 10 | ATTCATGCTA CCGGGCCGGC CGCCCAGCTG GGACTGTGCA  | 320  |
|    | CTTGCTGGTG GATGTTCCCC CCGAGGAGGC CCAGCTCTCC  | 360  |
|    | TGCTTCCGGA AGAGCCCCCT CAGCAATGTT GTTGTGAGT   | 400  |
|    | GGGGTCCCTCG GAGCACCCCA TCCCTGACGA CAAAGGCTGT | 440  |
| 15 | GCTCTTGGTG AGGAAGTTTC AGAACAGTCC GGCGAAGAC   | 480  |
|    | TTCCAGGAGC CGTGCCAGTA TTCCCAGGAG TCCCAGAAGT  | 520  |
|    | TCTCCTGCCA GTTAGCAGTC CCGGAGGGAG ACAGCTCTT   | 560  |
|    | CTACATAGTG TCCATGTGCG TCGCCAGTAG TGTCGGGAGC  | 600  |
| 20 | AAGTTCAGCA AAACTCAAAC CTTTCAGGGT TGTGGAATCT  | 640  |
|    | TGCAGCCTGA TCCGCCTGCC AACATCACAG TCACTGCCGT  | 680  |
|    | GGCCAGAAAC CCCCCGCTGGC TCAGTGTAC CTGGCAAGAC  | 720  |
|    | CCCCACTCCT GGAACTCATC TTTCTACAGA CTACGGTTTG  | 760  |
| 25 | AGCTCAGATA TCGGGCTGAA CGGTCAAAGA CATTACAAAC  | 800  |
|    | ATGGATGGTC AAGGACCTCC AGCATCACTG TGTCACTCCAC | 840  |
|    | GACGCCTGGA GCGGCCTGAG GCACGTGGTG CAGCTTCGTG  | 880  |
| 30 | CCCAGGAGGA GTTCGGGCAA GGCGAGTGGGA GCGAGTGGAG | 920  |
|    | CCCGGAGGCC ATGGGCACGC CTTGGACAGA ATCCAGGAGT  | 960  |
|    | CCTCCAGCTG AGAACGAGGT GTCCACCCCC ATGCAGGCAC  | 1000 |
| 35 | TTACTACTAA TAAAGACGAT GATAATATTC TCTTCAGAGA  | 1040 |
|    | TTCTGCAAAT GCGACAAGCC TCCCAGTGCA AGAT        | 1074 |

## (2) INFORMATION FOR SEQ ID NO:16:

40

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCGGATCC ATGCTGGGCA TCTGGACCCT CCTACC

36

55

(2) INFORMATION FOR SEO ID NO:17:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

15

AGCTTCGAGC GGCCGCGTTA GATCTGGATC CTTCCCTCTTT GC

42

20 (2) INFORMATION FOR SEQ ID NO:18:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 519 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

|    |  |                            |
|----|--|----------------------------|
| 35 | ATGCTGGCA TCTGGACCT CCTACCTCTG GTTCTTACGT<br>CTGTTGCTAG ATTATCGTCC AAAAGTGT TA ATGCCAAGT<br>GACTGACATC AACTCCAAGG GATTGGAATT GAGGAAGACT<br>GTTACTACAG TTGAGACTCA GAACCTGGAA GCCCTGCATC | 400<br>800<br>1200<br>1600 |
| 40 | ATGATGGCCA ATTCTGCCAT AAGCCCTGTC CTCCAGGTGA<br>AAGGAAAGCT AGGGACTGCA CAGTCATGG GGATGAACCA<br>GACTGCGTGC CCTGCCAAGA AGGGAAAGGAG TACACAGACA  | 200<br>240<br>280          |
| 45 | AAGCCCATT T TCTTCCAAA TGCGAGAGAT GTAGATTGTG<br>TGATGAAGGA CATGGCTTAG AAGTGGAAAT AAAC TGCACC<br>CGGACCCAGA ATACCAAGTG CAGATGTAAA CCAA ACTTTT  | 320<br>360<br>400          |
| 50 | TTTGTAAC TC TACTGTATGT GAACACTGTG ACCCTTGAC<br>CAAATGTGAA CATGGAATCA TCAAGGAATG CACACTCACC<br>AGCAACACCA AGTGCAGAAGA GGAAGGGATCC AGATCTAAC   | 440<br>480<br>519          |

55 (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

15           GCCRCCATGG

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## **Claims**

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1. A method of screening a plurality of compounds for the ability to bind a specific molecule comprising the steps:
  - a) contacting one or more compound with a chimeric protein containing two or more distinct domains wherein a first domain comprises at least a portion of said specific molecule or a peptide analog thereof and a second domain contains at least a portion of an immunoglobulin chain having one or more region selected from the group consisting of:
    - i) an epitope, and
    - ii) a immunoglobulin region able to recognize an epitope,
  - b) forming a binding partner complex between said chimeric protein and at least one of said compounds,
  - c) separating the complex from chimeric protein molecules not binding at least one compound,
  - d) contacting the binding partner complex with a directly or indirectly labeled secondary molecule able to bind the second domain of said chimeric protein, and
  - e) detecting said label as an indication of the presence of said compound.
2. The method of claim 1 wherein said first and second domain of said chimeric protein are separated by an immunoglobulin heavy chain hinge region.
3. The method of claim 1 or 2 wherein said specific molecule is selected from the group consisting of:
  - a) an antigen,
  - b) an antibody,
  - c) an enzyme,
  - d) an enzyme substrate,
  - e) a receptor, and
  - f) a ligand.
4. The method of claim 1 or 2 wherein said specific molecule is selected from the group consisting of: growth hormone, human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin-B chain, proinsulin, relaxin A-chain, leptin-receptor, fibroblast growth factor, relaxin B-chain, prorelaxin, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hormone, glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, tissue plasminogen activator, bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor alpha, tumor necrosis factor beta, enkephalinase, human serum albumin, mullerian-inhibiting substance, gonadotropin-associated peptide,  $\beta$  lactamase, tissue factor protein, inhibitin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- $\beta$ , platelet growth factor, transforming growth factor, TGF- $\alpha$ , TGF- $\beta$ , insulin-like growth factor I and II, insulin growth factor binding proteins, CD4, CD8, Dnase, Rnase, latency associated peptide, eryth-

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ropoietin, osteoinductive factors, interferon-alpha, -beta and -gamma, colony stimulating factors, M-CSF, GM-CSF, G-CSF, stem cell factor, interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, superoxide dismutase, viral antigens, HIV envelope proteins, gp120, gp140, immunoglobulins, and proteins encoded by the Ig supergene family, and the naturally-occurring ligands or receptors of these compounds.

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5. The method of claim 4 wherein said specific molecule comprises at least a portion of the tumor necrosis factor alpha receptor.

6. The method of claim 4 wherein said specific molecule comprises at least a portion of the endothelial growth factor receptor.

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7. The method of claim 4 wherein said specific molecule comprises at least a portion of the thrombopoietin receptor.

8. The method of claim 4 wherein said specific molecule comprises at least a portion of the TGF alpha receptor.

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9. The method of claim 4 wherein said specific molecule comprises at least a portion of the TGF beta receptor.

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10. The method of claim 4 wherein said specific molecule comprises at least a portion of the erythropoietin receptor.

11. The method of claim 4 wherein said specific molecule comprises at least a portion of the interferon gamma receptor.

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12. The method of claim 4 wherein said specific molecule comprises at least a portion of the GM-CSF receptor.

13. The method of claim 4 wherein said specific molecule comprises at least a portion of the G-CSF receptor.

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14. The method of claim 4 wherein said specific molecule comprises at least a portion of the IL-4 receptor.

15. The method of claim 4 wherein said specific molecule comprises at least a portion of the IL-6 receptor.

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16. The method of claim 4 wherein said specific molecule comprises at least a portion of the leptin receptor.

17. The method of claim 4 wherein said specific molecule comprises at least a portion of the fibroblast growth factor receptor.

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18. The method of claim 2 wherein said first domain is positioned to the amino terminal side of said second domain on said chimeric protein.

19. The method of claim 2 wherein said first domain is positioned to the carboxy terminal side of said second domain on said chimeric protein.

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20. The method of claim 18 wherein said immunoglobulin portion of said second domain comprises the C<sub>H</sub>3 region of an immunoglobulin heavy chain.

21. The method of claim 20 wherein said immunoglobulin portion of said second domain comprises the C<sub>H</sub>2 region of an immunoglobulin heavy chain.

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22. The method of claim 1 or 2 wherein said compounds are immobilized on a solid support.

23. The method of claim 1, 2 or 18 wherein said compounds comprise at least a portion of a chemical combinatorial library.

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24. The method of claim 23 wherein said library is comprised of members of the group selected of:

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a) naturally-occurring or non-naturally occurring amino acids,  
b) naturally-occurring or non-naturally occurring nucleotides,  
c) naturally-occurring or non-naturally occurring saccharides, and  
d) bi- or multifunctional small organic molecules.

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25. The method of claim 22 wherein step c) is accomplished by washing the solid support free of uncomplexed chimeric protein.

5 26. The method of claim 1 or 2 wherein said chimeric protein is produced by expression, within a host cell, of a recombinant DNA open reading frame encoding said chimeric protein.

27. The method of claim 26 wherein said host cell expresses said chimeric protein as a dimer joined by at least one disulfide linkage, said dimer containing at least two specific binding partners.

10 28. The method of claim 22 wherein said compounds are contacted with bivalent chimeric protein dimers containing at least two specific binding partners.

29. The method of claim 26 wherein said host cell expresses DNA containing a second open reading frame encoding a second chimeric protein, said second chimeric protein comprising a first domain containing at least a portion of said specific molecule or an analog thereof, and a second domain comprising at least a portion of an immunoglobulin chain having a region selected from the group consisting of:

15 i) an epitope, and  
ii) a immunoglobulin region able to recognize an epitope,

20 wherein said second chimeric protein contains at least a portion of an immunoglobulin light chain.

30. The method of claim 29 wherein said chimeric protein and said second chimeric protein are comprised in a multimeric complex linked by at least one disulfide bond.

25 31. The method of claim 30 wherein the first domains of said chimeric protein and said second chimeric protein contain the same specific molecule portion or peptide analog thereof.

30 32. The method of claim 30 wherein the first domains of said chimeric protein and said second chimeric protein contain different specific molecule portions or peptide analogs thereof.

35 33. The method of claim 28 wherein at least one of said compounds are present in the form of a multimer, and said linked fusion protein dimer binds said compound more strongly than does a monomeric chimeric protein alone.

34. The method of claim 30 wherein at least one of said compounds are present in the form of a multimer and said multimeric complex binds said compound more strongly than do either said first or second chimeric protein alone.

35 35. The method of claim 26 wherein said host cell is a eukaryotic cell.

40 36. The method of claim 29 wherein said host cell is a eukaryotic cell.

37. The method of claim 26 wherein said open reading frame contains nucleotide sequences which direct the cell to add N-linked sugar residues to the chimeric protein expressed therefrom.

45 38. The method of claim 2 wherein said solid support is a cell.

39. The method of claim 2 wherein said solid support is a bacteriophage particle.

40. A method for screening one or more compounds for the ability to bind a specific molecule comprising the steps:

50 a) immobilizing to a solid support a chimeric protein containing two or more distinct domains wherein a first domain comprises at least a portion of said specific molecule or a peptide analog thereof and a second domain contains at least a portion of an immunoglobulin chain having a region selected from the group consisting of:

55 i) an epitope, and  
ii) a immunoglobulin region able to recognize an epitope,

wherein said chimeric protein is immobilized to the solid support by an interaction between said solid support

and said second domain,

- b) contacting the immobilized chimeric protein with said compound or compounds to form a binding partner complex between the chimeric protein and compounds able to bind the specific molecule,
- c) washing said solid support to separate the complex from chimeric protein molecules not binding at least one compound,
- d) detecting said chimeric protein as an indication of the presence of said compound.

5 41. The method of claim 40 wherein said first and second domain of said chimeric protein are separated by an immunoglobulin heavy chain hinge region.

10 42. The method of claim 41 wherein said first domain is positioned to the amino terminal side of said second domain on said chimeric protein.

15 43. The method of claim 41 wherein said first domain is positioned to the carboxy terminal side of said second domain on said chimeric protein.

44. The method of claim 42 wherein said immunoglobulin portion of said second domain comprises the C<sub>H</sub>3 region of an immunoglobulin heavy chain.

20 45. The method of claim 44 wherein said immunoglobulin portion of said second domain comprises the C<sub>H</sub>2 region of an immunoglobulin heavy chain.

46. The method of claim 40 or 41 wherein said immobilized chimeric protein is in the form of a disulfide-linked multimeric complex.

25 47. The method of claim 46 wherein said multimeric complex binds to two or more sites of said compound or compounds.

30 48. The method of claim 40 or 41 wherein said compounds are comprised of members selected from the group consisting of:

- a) naturally-occurring or non-naturally-occurring amino acids,
- b) naturally-occurring or non-naturally-occurring nucleotides,
- c) naturally-occurring or non-naturally occurring saccharides, and
- d) bi- or multifunctional small organic molecules.

35 49. The method of claim 40 wherein said chimeric protein is immobilized by a binding interaction between said chimeric protein and a moiety joined to the solid support selected from the group consisting of:

- a) an antigen,
- b) at least a portion of an antibody,
- c) Protein G, and
- d) Protein A.

40 50. The method of claim 49 wherein said compound is eluted from said solid support before step d).

45 51. The method of claim 40 or 41 wherein the specific molecule is selected from the group consisting of:

- a) an antigen,
- b) an antibody,
- c) an enzyme,
- d) an enzyme substrate,
- e) a receptor, and
- f) a ligand.

50 55 52. The method of claim 40 or 41 wherein said specific molecule is selected from the group consisting of: growth hormone, human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin-B chain, proinsulin, relaxin A-chain, leptin receptor, fibroblast growth factor, relaxin β-chain, prorelaxin, follicle

stimulating hormone, thyroid stimulating hormone, luteinizing hormone, glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, tissue plasminogen activator, bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor alpha, tumor necrosis factor beta, enkephalinase human serum albumin, mullerian-inhibiting substance, gonadotropin-associated peptide,  $\beta$  lactamase, tissue factor protein, inhibitin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- $\beta$ , platelet growth factor, transforming growth factor, TGF- $\alpha$ , TGF- $\beta$ , insulin-like growth factor I and II, insulin growth factor binding proteins, CD4, CD8, Dnase, Rnase, latency associated peptide, erythropoietin, osteoinductive factors, interferon-alpha, -beta and -gamma, colony stimulating factors, M-CSF, GM-CSF, G-CSF, stem cell factor, interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, superoxide dismutase, viral antigens, HIV envelope proteins, gp120, gp140, immunoglobulins, and proteins encoded by the Ig supergene family, and the naturally-occurring ligands, receptors, and/or substrates of these compounds.

53. A method of screening a compound for the ability to bind a specific binding partner comprising the steps:

15       a) constructing a recombinant DNA vector able to be expressed in a host cell, which vector comprises:

20       i) an open reading frame containing a first sequence region encoding at least a portion of an immunoglobulin chain which immunoglobulin chain contains one or more region selected from the group consisting of a region able to bind to an antigen, a region able to bind to an antibody, and an immunoglobulin-derived hinge region, and  
ii) a promoter sequence positioned upstream of said open reading frame and able to direct RNA transcription of said open reading frame within said host cell,  
25       wherein said open reading frame contains at least one restriction site located between said first sequence region and said promoter sequence for cloning a second nucleotide sequence region encoding at least a portion of a specific binding partner, provided said first and second nucleotide sequence region are cloned so as to preserve said open reading frame between said promoter sequence and a stop codon located not before the 3' end of said first nucleotide sequence region,

30       b) inserting said second nucleotide sequence into the vector at said restriction site,  
c) causing said vector to enter said host cell,  
d) incubating said host cell under conditions causing the expression of a chimeric protein containing the amino acids encoded by said first and second nucleotide sequence,  
e) separating said chimeric protein from said host cell,  
35       f) contacting the compound with said chimeric protein under conditions favoring the binding of said compound with said specific binding partner portion of the chimeric protein, and  
g) specifically detecting the presence of a bound fusion protein:compound complex as an indication of the presence of compounds able to bind said specific binding partner.

40       54. The method of claim 53 wherein a third nucleotide sequence region encoding at least a portion of the hinge region of an immunoglobulin heavy chain is positioned between said first and second sequence region so as to preserve said open reading frame between said promoter sequence and a stop codon located at or near the 3' end of said first nucleotide sequence region.

45       55. The method of claim 53 wherein said open reading frame encodes, upon expression, a chimeric protein containing two or more distinct domains wherein a first domain comprises at least a portion of a specific binding partner and a second domain contains at least a portion of an immunoglobulin chain having a region selected from the group consisting of:

50       i) an epitope, and  
ii) a immunoglobulin region able to recognize an epitope.

56. The method of claim 55 wherein said specific binding partner will bind a member of the group consisting of:

55       a) an antigen,  
b) an antibody,  
c) an enzyme,  
d) an enzyme substrate,

e) a receptor, and  
f) a ligand.

57. The method of claim 56 wherein said specific binding partner will bind at least a portion of a compound selected from the group consisting of: growth hormone, human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin-B chain, proinsulin, relaxin A-chain, leptin receptor, fibroblast growth factor, relaxin B-chain, prorelaxin, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hormone, glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, tissue plasminogen activator, bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor alpha, tumor necrosis factor beta, enkephalinase human serum albumin, mullerian-inhibiting substance, gonadotropin-associated peptide,  $\beta$  lactamase, tissue factor protein, inhibitin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- $\beta$ , platelet growth factor, transforming growth factor, TGF- $\alpha$ , TGF- $\beta$ , insulin-like growth factor I and II, insulin growth factor binding proteins, CD4, CD8, Dnase, RNase, latency associated peptide, erythropoietin, osteoinductive factors, interferon-alpha, -beta and -gamma, colony stimulating factors, M-CSF, GM-CSF, G-CSF, stem cell factor, interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, superoxide dismutase, viral antigens, HIV envelope proteins, gp120, gp140, immunoglobulins, and proteins encoded by the Ig supergene family, the naturally-occurring ligands, receptors, and/or substrates of these compounds, and analogs of these compounds, receptors and substrates thereof.

58. A method of screening a compound for the ability to bind a specific binding partner comprising the steps:

- constructing a recombinant DNA vector able to be expressed in a host cell, which vector comprises:
  - an open reading frame containing a first sequence region encoding at least a portion of an immunoglobulin chain, and
  - a promoter sequence positioned upstream of said open reading frame and able to direct RNA transcription of said open reading frame within said host cell,  
wherein said open reading frame contains at least one restriction site located at or near the 3' end of the first sequence region for cloning a second nucleotide sequence region encoding at least a portion of a specific binding partner, provided said first and second nucleotide sequence region are cloned so as to preserve said open reading frame between said promoter sequence and a stop codon located not before the 3' end of said second nucleotide sequence region,
  - inserting said second nucleotide sequence into the vector at said restriction site,
  - causing said vector to enter said host cell,
  - incubating said host cell under conditions causing the expression of a chimeric protein containing the amino acids encoded by said first and second nucleotide sequence,
  - separating said chimeric protein from said host cell,
  - contacting said compound with said chimeric protein under conditions favoring the binding of said compound with said specific binding partner portion of the chimeric protein, and
  - specifically detecting the presence of a bound fusion protein:compound complex as an indication of the presence of compounds able to bind said specific binding partner.

59. The method of claim 58 wherein a third nucleotide sequence region encoding at least a portion of the hinge region of an immunoglobulin heavy chain is positioned between said first and second sequence region so as to preserve said open reading frame between said promoter sequence and a stop codon located at or near the 3' end of said second nucleotide sequence region.

60. The method of claim 59 wherein said first nucleotide region open reading frame encodes, upon expression, a chimeric protein containing two or more distinct domains wherein a first domain comprises at least a portion of a specific binding partner and a second domain contains at least a portion of an immunoglobulin chain having a region selected from the group consisting of:
 

- an epitope, and
- a immunoglobulin region able to recognize an epitope.

61. The method of claim 60 wherein said first nucleotide sequence region encodes at least a portion of an immunoglobulin variable region.

62. The method of claim 60 or 61 wherein said specific binding partner portion will bind a member of the group consisting of:

- 5      a) an antigen,
- b) an antibody,
- c) an enzyme,
- d) an enzyme substrate,
- e) a receptor, and
- f) a ligand.

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63. The method of claim 60 or 61 wherein said specific binding partner will bind at least a portion of a compound selected from the group consisting of: growth hormone, human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin-B chain, proinsulin, relaxin A-chain, leptin receptor, fibroblast growth factor, relaxin B-chain, prorelaxin, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hormone, glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, tissue plasminogen activator, bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor alpha, tumor necrosis factor beta, enkephalinase human serum albumin, mullerian-inhibiting substance, gonadotropin-associated peptide,  $\beta$  lactamase, tissue factor protein, inhibitin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- $\beta$ , platelet growth factor, transforming growth factor, TGF- $\alpha$ , TGF - $\beta$ , insulin-like growth factor I and II, insulin growth factor binding proteins, CD4, CD8, Dnase, Rnase, latency associated peptide, erythropoietin, osteoinductive factors, interferon-alpha, -beta and -gamma, colony stimulating factors, M-CSF, GM-CSF, G-CSF, stem cell factor, interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, superoxide dismutase, viral antigens, HIV envelope proteins, gp120, gp140, immunoglobulins, and proteins encoded by the Ig supergene family, the naturally-occurring ligands, receptors, and/or substrates of these compounds, and analogs of these compounds, receptors and substrates thereof.

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